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A Quarterly Journal of Zoölogical Research

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STUDIES ON PROTOZOA, IV: LETHAL EFFECTS OF THE X-RADIATION OF A STERILE CULTURE MEDIUM FOR COLPIDIUM CAMPYLUM[1]

(One figure)

C. V. TAYLOR, J. O. THOMAS, AND M. G. BROWN
Stanford University

IN OUR collaborative studies on the effects of very intense soft X-rays on various Protozoa, reproducible control of the culture medium has been considered necessary for any attempt successfully to repeat and rightly interpret our results. To this end extensive preliminary investigations were undertaken on the nutritional requirements of several ciliates, some results of which have now been published (Luck, Sheets, and Thomas, 1931; Luck and Sheets, 1931; Brown, Luck, Sheets, and Taylor, 1933).

The utilization of a sterile 10 per cent tap-water solution of yeast extract[2] for the culture of *Colpidium campylum*[3] has thus far met with good success. Under ordinary laboratory conditions pure strains of this holotrichous ciliate washed free of bacteria have thrived vigorously for about two years in this convenient and reproducible sterile medium.

[1] Contribution No. 4 from the Laboratories of Chemophysical Biology. These investigations were generously supported through a grant from the National Research Council's Committee on Irradiation.

[2] Prepared by mixing 450 gms. of a commercial yeast with 500 cc. of water. This was kept at 50° C. for 24 hours, then neutralized, filtered and autoclaved. For cultures, the filtrate was diluted 10 times with tap water.

[3] The specific identity of this ciliate appears to us not yet established.

Subsequent to investigations to determine the lethal dosage of the intense soft X-rays on this ciliate, it was decided to test for any effects the X-radiated medium itself might have on *unexposed* Colpidia. For example, a small quantity (0.05 cc.) of freshly prepared yeast extract was irradiated 150 seconds receiving a dosage of 30×10^4 Roentgen units, and inoculated with the ciliates immediately or at a given time later: into each such quantity was introduced by sterile mouth-pipette from 5 to 10 Colpidia with a trace of their unradiated culture medium. Controls were run throughout.

The results were striking. About three hours later all of the ciliates on each slide containing the X-radiated medium had disintegrated, leaving in evidence for each *Colpidium* a conspicuous cluster of its granular inclusions. The controls were normal.

Further tests for this lethal effect on the Colpidia were confirmatory. We undertook, therefore, to ascertain what causal agent or agents were primarily responsible for the death of the organisms. It was soon discovered that the X-radiation merely of the tap water which was thereupon used in preparing the yeast medium rendered that medium toxic to the Colpidia. Colorimetric tests with titanic chloride specifically demonstrated in all of 18 cases the presence of H_2O_2 in the irradiated water.

It became apparent, however, that distilled or tap water which had been freed of air by boiling, upon being X-radiated for an equivalent dose did not yield H_2O_2 in any amounts detectable with the titanic chloride (which is sensitive to 1 part of H_2O_2 in more than one million parts of water) and such water was not toxic to the ciliates. On the other hand, the X-radiation, at an equivalent dosage, of air-free, oxygenated water, saturated at 20° C, and at atmospheric pressure, did produce H_2O_2, and in concentrations which proved toxic to the ciliates. From colorimetric comparisons with known dilutions of Merck's H_2O_2, the concentration of the peroxide in this irradiated water was estimated to be about 1:90,000.

MATERIAL AND METHOD

A. THE CULTURE MEDIUM

The holotrichous ciliate, *Colpidium campylum*, was used as the experimental organism throughout these investigations. It was obtained originally in an enrichment culture from spinach procured

in the vicinity of Monterey Bay and sub-cultured in a continuously thriving condition in a sterile tap-water extract of commercial yeast. The extract as prepared was diluted tenfold, then apportioned in 5 cc. amounts into test tubes. Sterile technique was used throughout. Each tube was inoculated with approximately 1000 Colpidia. Resulting growth was vigorous and uniform.

In the experiments described below, 4-day old cultures of the ciliate in a 10 *per cent dilution* of the extract were employed. At this dilution the organisms still grow exceedingly well, and have continued to thrive vigorously now for more than 2 years.

It is obvious that this successful and convenient method of culture of this ciliate in the absence of bacteria affords unique control of the food factor which for experimental purposes is of first importance. Our findings of the lethal effects of the X-radiated medium on the Colpidia, recorded in later paragraphs, were made possible, we believe, through the use of this reproducible medium. It now appears that the method opens a fruitful field not only for irradiation studies but also for various other fundamental investigations on the vital activities of this unicellular organism.

The medium to be X-radiated, with or without organisms, was placed on the floor of the depression of a hanging-drop slide and covered by a thin sheet of mica of known thickness[4] sealed on with sterile vaseline.

With an initial inoculum of approximately 1000 individuals in 5 cc. of the 10 per cent yeast medium, the population density in 4 days is such that 1 drop of the culture (delivered from the standard micropipette employed in these experiments) contained from 5 to 10 Colpidia. The volume of this "standard" drop was 2.5×10^{-3} cc., which is 5 per cent of the 0.05 cc. of X-radiated medium to which the drop was afterward added. This drop method of adding the sterile Protozoa, washed or unwashed, to the depression slide has been used throughout the experiments to be described.

B. X-RAY EQUIPMENT[5]

The X-ray equipment now being used in our laboratories was especially designed and built by Dr. Harry Clark with the assistance

[4] The thickness of the mica coverslips was accurately measured in the Stanford Gauge Laboratory through the kindness of Mr. M. S. Hugo.

[5] A full description of the equipment is expected to be published in the near future.

of M. G. Brown. Its very careful plan and simplified construction for the production of exceedingly intense soft X-rays account for its remarkable efficiency and constant, precise control in operation.

The construction features which give high intensity of X-ray ionization within the specimen under study are a small dimension X-ray tube, a thin window, and an efficient cooling system. The X-ray tube is of metal construction throughout except for a straight section of glass tubing to insulate the anode. This metal construction permits the use of small dimensions, the only limit being that required to prevent field currents. The distance between smooth surfaces of the anode and the grounded cathode or tube walls is $6\frac{1}{2}$ mm. which is sufficient for the 50,000 volts maximum for which this tube has been designed. The distance from the focal spot of the silver target to the drop of media being irradiated has been reduced to but 3 centimeters. Metal construction also permits water cooling of the tube and so the environs of the specimen are kept at room temperature. The X-rays escape from the tube by way of a 0.05 mm. aluminium window. This window transmits a large fraction of the soft X-rays and completely absorbs the visible rays from the incandescent cathode as well as the heat rays which are conducted to the water-cooled part of the tube. Provision has also been made for cooling this thin window by means of an air blast, but this has been found unnecessary.

In order that the X-ray intensity and quality may be carefully controlled, the high voltage is obtained by means of a transformer whose output is rectified by a pair of kenetrons and smoothed out by choke coils and a large condenser. This condenser is of sufficient capacity to maintain a potential whose fluctuation in kilovolts is calculated to be not more than 0.30 times the current in milliamperes. The voltage has been measured by means of an accurate electrostatic voltmeter[6] and is calibrated in terms of the primary voltage of the high-voltage transformer for various loads. The voltage is kept practically constant by a variable resistance in the primary circuit.

The X-ray ionization intensity is measured by means of an air

[6] For a detailed description see the article by Dr. Harry Clark in *Review of Scientific Instruments*, 1, No. 11 (1930).

ionization chamber whose ionization current is determined by balancing the charge collected on the insulated plate of the chamber by turning a variable condenser of known capacity. Turning the condenser as the charge collects keeps the insulated system at ground potential as determined by observation of a built-in gold-leaf electroscope. In this way there can be no error due to a distorted field within the ionization chamber.

Throughout these experiments the X-ray tube has been operated at 45,000 volts and 30 milliamperes. This power is largely dissipated by water cooling the anode so that continuous operation for a period of several hours may be maintained. The intensity varied only with the kind and thickness of the walls inclosing the specimen under irradiation. In all irradiations of small portions of media, the intensity has been corrected for the absorption of the mica coverslip.

EXPERIMENTAL

A. PRELIMINARY X-RADIATION OF *Colpidium campylum*

During the spring and summer of 1931, we undertook to establish lethal dose curves for this ciliate, cultured in the sterile yeast solution, by X-radiating the free-living organism in the medium and plotting dosages of X-radiation against percentages of survivors at given intervals after the exposure. While it was determined that a dosage of 46×10^4 Roentgen units would produce death of the ciliates *within 15 minutes following exposure*, the curves were unsatisfactory and it was decided to investigate any possible effects the X-radiated medium might have upon the unexposed organisms.

B. X-RADIATION OF THE YEAST MEDIUM AND SUBSEQUENT ADDITION OF *Colpidium campylum*

It readily became apparent that the irradiation of the yeast medium to which Colpidia from a thriving sterile culture were subsequently added rendered that medium lethal to the organisms. This induced toxicity of the medium was all the more striking because the dose thus required was only slightly greater than the dosage needed to kill the Protozoa when irradiated along with the medium. This comparison, it should be noted, refers here as in later paragraphs to *ultimate* death of the Colpidia. In the above instance,

for example, although a dose of 46×10^4 R. units caused death within 15 minutes following the exposure, the dosage required to effect ultimate death was actually less as experiments below demonstrate.

Confirmation of these striking results followed upon further experiments so completely that we set out to discover, if possible, the lethal factor or factors involved and their immediate source. It was evident that the lethal state of the yeast medium might be induced either indirectly by the ionizing action of the X-rays on (*a*) the glass or (*b*) the air adjacent to the exposed medium, or directly within the medium itself. Experiments were accordingly planned to test these several possibilities.

C. POSSIBLE INDIRECT EFFECTS UPON THE MEDIUM FROM X-RADIATED GLASS ADJACENT TO IT

The brown or purple coloration of glass from exposure to X-rays is a well-known but little understood phenomenon. It seemed not unlikely that this conspicuous change in our exposed glass depression slides might be accompanied by a liberation of toxic substances from the glass into the contained medium. In order to test this, we compared relative effects of equivalent dosages of the X-rays on (*a*) a given volume of the medium placed directly on the floor of the depression slide, and (*b*) an equal volume of that medium which was protected from the glass by a layer of paraffin of given thickness on the floor of the depression. A third comparison was made for equally exposed medium of like volume which was delimited in the depression by a ring of paraffin of given diameter ($2\frac{1}{2}$ mm). The ring was cut with a microtome from a hollow cylinder of paraffin and fixed to the floor of the depression by careful warming. A constancy in the diameter, depth, and exposure surface of the medium was thus insured by the paraffin ring and a condition intermediate between that of a drop resting entirely on glass and entirely on paraffin was thus attained. The volume of the yeast extract X-radiated in each case was 0.05 cc. *Within one minute after the exposure*, upon removing the mica cover, 1 droplet of unexposed medium (approximately 2.5×10^{-3} cc.) containing from 5 to 10 Colpidia from a 4-day old culture, was added to the X-radiated 0.05 cc. and the cover

sealed on again with sterile vaseline. The amount of non-irradiated medium thus added was probably negligible.

All the slides in any one series were made up together under identical conditions and irradiated within a few hours of each other. Following the inoculation with Colpidia, observations of their reactions to the irradiated medium were made from time to time up to their ultimate death or recovery. The organisms were observed to die ordinarily from one-half to three hours after their inoculation. It should be noted also that the irradiated medium of some slides marked "viable" in Table I was perceptibly toxic to the introduced Colpidia which exhibited groggy swimming movements for some time after inoculation.

Some of the series in Table I show a lethal dose for plain slides to be smaller than that for paraffined slides. This, however, may be attributed mainly to the broader surface exposed and the correspondingly shallower depth of medium in the plain slides, which would allow relatively greater X-ray activation within the medium itself. This interpretation need not, of course, rule out some indirect effect such as due to a diffusion of toxic substances into the medium from the adjacent X-rayed glass. In any case, the differences are not as marked as might be expected if the chief effect on the medium were by way of the glass.

D. THE DELAYED INOCULATION OF COLPIDIA INTO THE X-RADIATED STERILE MEDIUM IN PLAIN AND IN PARAFFINED SLIDES

It was thought that an analysis of the lethal factors involved in the X-radiated medium might be facilitated through testing their stability. It appeared also that some differentiation in the results might be discovered by using, as in foregoing experiments, both plain and paraffined slides. It was evident, from delayed inoculation of the Colpidia into the X-rayed medium, that meantime the medium had recovered to a less toxic condition. This is illustrated in Table I by Series 25 in which a delay of 6 hours showed definitely a decreased toxicity.

E. DETECTION OF A LETHAL AGENT PRODUCED IN X-RADIATED TAP AND DISTILLED WATER

As a result of X-radiation, the highly proteinaceous components of yeast extract might conceivably yield certain organic products,

TABLE I[7]

RESULTS OF THE IRRADIATION OF YEAST EXTRACT IN PLAIN AND PARAFFINED SLIDES AND OF EFFECTS OF DELAYED INOCULATION

Symbols: G, glass depression slides without paraffin; P, depression slide with paraffin layer covering bottom; PG, depression slide with a ring of paraffin sealed to bottom; o, viable Colpidia; ×, their ultimate death.

Series	Hours Delay in Inoculation	Type of Slide	X-Ray Dose in Minutes										
			0	1	2	3	4	5	6	7	8	9	10
11	0	P	o	o o o	o	o ×	× × ×	× ×	× × ×				
23	0	G	o				× ×			× ×			× ×
	12	G	o				× ×			× ×			× ×
25	0	G	o	o	o	×	×			×			
	6	G	o	o	o	o	o			×			
27	0	G	o		o	o	×			×		×	
	0	PG	o				o			×		×	
28	0	G	o		o	×	o ×	×		×			
	0	PG	o			×	× ×	×		×		×	
30	0	G	o		×	× × × ×	×						
	0	PG	o	o o	o × ×	×	× × ×						
32	0	G	o	×	× ×	× × × × ×							
	0	PG	o o	o	×	× o	× × ×	× ×					
34	0	G	o o	o	o	× × ×	× ×						
	0	PG	o	o o o	o o o	o × ×	× ×						

[7] Five series have been omitted from this table owing to inconsistencies within the series which were believed to be due to faulty technique.

such as phenols, which could account for the toxicity of our irradiated yeast medium. But due to the obvious difficulties involved in a satisfactory determination of possible organic toxic substances, we undertook first to detect in the constituent water itself any inorganic substance that might be responsible for the lethal effects observed. To this end the following experiments were performed:

1. Nine depression slides containing yeast medium (as noted above) were exposed to X-ray dosages from 26.5 to 46.9×10^4 R. To this irradiated medium were added from 5 to 10 Colpidia immediately after the exposure. Within 19 hours all the Colpidia in each of the 9 slides had died.

2. Similarly, 9 depression slides containing only tap water were prepared and X-radiated in dosages from 26.2 to 47.9×10^4 R. Thereupon, after yeast extract had been added in a final concentration of 10 per cent, each slide was inoculated with from 5 to 10 Colpidia. Similar results followed: after 19 hours all the Colpidia in each of the 9 slides had died, but the controls showed normal behavior and usual rate of reproduction.

These experiments showed, then, that in order to render the yeast medium lethal to the Colpidia it was sufficient to X-radiate only the tap water to be used in making up that medium. It was not clear, of course, to what extent the same factor or factors operated lethally in both cases: (1) that of the X-radiated medium on the one hand, and (2) that of the X-radiated tap water on the other. But the evidence for induced lethal substance or substances in the irradiated tap water appeared positive enough to warrant an intensive search for their identification.

At this point it may be noted that a cursory review of protozoölogical literature on X-ray studies did not yield any help in our endeavor. Analogous results of investigations by Bovie and Hughes, 1918; Brooks, 1918, and others using ultra-violet radiation were encouraging. But it was not until after we had definitely identified in several sorts of X-ray experiments with both tap and distilled water an important lethal agent, hydrogen peroxide, that extensive recourse was made to the physical literature. Here the recent work of Risse (1929*a*) proved most helpful. This especially will be referred to later under the caption "Discussion."

F. IDENTIFICATION OF HYDROGEN PEROXIDE IN X-RADIATED WATER

The methods employed for the identification of one important lethal agent in X-radiated tap and distilled water were direct and relatively simple. This lethal substance proved to be hydrogen peroxide, the specific test for which was the exceedingly sensitive reagent, titanic chloride (Barnaby, 1910; Risse, 1925*b*, and Ato, 1930).

The following are considered to be the reactions involved:

(1) $TiCl_4+4H_2O \rightarrow Ti(OH)_4+4HCl$
(2) $Ti(OH)_4 \rightarrow TiO_2+2H_2O$
(3) $TiO_2+H_2O_2 \rightarrow H_2O+Ti\,O_3$

The pertitanic anhydride (TiO_3)) thus formed imparts an orange color to the solution. With Merck's 30 per cent H_2O_2 we find the test to be sensitive to 1 part in at least 2,000,000.

TABLE II

Slide Number	X-ray dosage at 20° C.	Test with 1.0 NTiCl$_4$
1	400×10^4 R.	Positive
2	400×10^4 R.	Positive
3	700×10^4 R.	Positive
4	800×10^4 R.	Positive

Using titanic chloride as the specific reagent for hydrogen peroxide, the following experiments were performed:

1. 0.15 cc. portions of triple-distilled water were placed in 4 depression slides and covered with mica cover slips of known thickness which were sealed down with paraffin. After the X-radiation 4 drops (approximately 1×10^{-3} cc.) of 1.0 NTiCl$_4$ were added to each of the 4 portions, the results being shown in Table II.

The tests for H_2O_2 were carried out immediately after the irradiation by transferring the X-rayed water from each slide respectively to 4 small glass tubes (inside diameter $2\frac{1}{2}$ mm.) and adding to each the 4 drops of 1.0 NTiCl$_4$. A light amber color readily appeared in each tube. The controls remained colorless. While it was evident that H_2O_2 was present in the 4 tubes containing the X-radiated water, no reliable estimate can be offered as to its quantity. A crude

comparator, constructed for these tests, proved quite helpful qualitatively.

2. Since the triple-distilled water employed in the above experiment (1) had for some days been freely exposed to air, we decided to test for H_2O_2 in air-free but *oxygenated* distilled water. To this end triple-distilled water was boiled, then cooled to 5° C., whereupon O_2 was bubbled through it, allowing ample time for saturation. This oxygenated water was then X-radiated at 20° C. and the test for H_2O_2 was made as in (1) above, with the results as shown in Table III.

The color in each tube was conspicuously more intense than in the series of (1). The controls were again colorless. We estimated

TABLE III

Slide	0.15 cc. Portion X-Rayed at 20° C.	Test with 1.0 NTiCl$_4$
1	800×10^4 R.	Pronounced color
2	700×10^4 R.	Pronounced color
3	400×10^4 R.	Pronounced color
4	200×10^4 R.	Pronounced color

the concentration of H_2O_2 in this series (compared with known dilutions of Merck's H_2O_2) to be roughly 1:50,000.

3. In order to maintain the oxygenated water, during the period of its irradiation, free from any exposure to the air, a flattened glass ampule (Pyrex) was drawn so that a long capillary extended from each end. The ampule, having a volume of approximately 0.5 cc., was then filled by suction with twice-distilled water which had been boiled, cooled to 5° C., and oxygenated as in (2) above. The capillaries were thereupon instantly sealed off by means of an oxygen-gas micro-flame. When the contained water cooled, a small bubble of water vapor (as indicated by its decrease in size upon warming) appeared in the ampule. The dosage of X-rays then applied was about 200×10^4 R.[8] Upon removing this irradiated water and immediately testing with 1.0 NTiCl$_4$ for hydrogen peroxide, a pronounced yellow color appeared.

[8] This dosage is rather approximate since the absorption in the glass was not accurately known.

4. Two ampules of capacity about 1 cc. each, such as described in (3), above, were filled with tap water which had previously been boiled, cooled to 5° C. and oxygenated. Each ampule was likewise sealed off by means of the micro-flame.

After irradiation, approximately half of the water was removed from each of the 2 ampules and tested for H_2O_2 with positive results, as Table IV indicates.

The remaining 0.5 cc. portion from each ampule was then used to prepare a culture of *Colpidium campylum*. Several ciliates were introduced directly upon adding yeast extract to each portion to a final concentration of 10 per cent. Five hours later these organisms all exhibited slow, groggy movements. On the following morning,

TABLE IV

Ampule	Approximate X-Ray Dosage at 23° C.	Test with 1.0 $N TiCl_4$
1	120×10^4 R.	Pronounced yellow color
2	220×10^4 R.	Pronounced yellow color

however, the ciliates had apparently fully recovered. From data to be presented in later paragraphs it appears fairly probable that the recuperation of these Colpidia may be attributed in part to the dilution of H_2O_2 formed and to the presence of the yeast extract which, it will be noted, was freshly added to the X-radiated water. Certain findings of Glocker and Risse (1928), which will be discussed later, regarding the decomposition of H_2O_2 by X-rays, also have a bearing on this point.

G. THE ABSENCE OF HYDROGEN PEROXIDE IN OXYGEN-FREE X-RADIATED WATER

Having found the production of X-rays of hydrogen peroxide in both aerated and oxygenated water in concentrations highly toxic or lethal to Colpidia, it occurred to us that this induced formation of H_2O_2 might depend upon the presence of the dissolved oxygen in the X-radiated water. As yet we were unaware of the recent excellent researches of Risse (1929*a*, 1929*b*, 1930), Glocker and Risse (1928), and of Fernau (1923), on this important problem, but since its solu-

tion would affect so significantly the interpretation of our previous findings, we performed with the excellent equipment available the 3 following experiments toward that end:

1. Two glass ampules of approximately 1 cc. capacity were prepared as described in a preceding paragraph and filled by suction with triple-distilled water which had been boiled for about 30 minutes. Their capillaries were then sealed off with the microtorch. Immediately following the X-radiation the water was removed from each ampule and tested in the comparator tubes for any hydrogen peroxide. Positive tests were obtained (see Table V).

The results suggested that H_2O_2 can be produced by X-rays in oxygen-free water. But the likelihood of some oxygen remaining

TABLE V

Ampule	X-Ray Dosage (Approximate)	Test for H_2O_2 with 1.0 $N TiCl_4$
1	300×10^4	Perceptible color
2	200×10^4	Perceptible color

on and in the walls of the ampules urged an improvement in our technique. It was clearly essential that a method of utmost precision be employed in order to eliminate oxygen from both the water and the glass container.

2. Accordingly, an especially designed triangle with accessory parts was constructed of Pyrex glass, similar to that illustrated in Figure 1, and sealed to a mercury diffusion pump. This triangle, together with its accessories, which included a charcoal tube, was enclosed in an insulated oven and there baked at 500° C. during evacuation. The baking and evacuation were continued over a period of 5 consecutive days until a vacuum of approximately 1×10^{-5} mm. Hg was obtained. At this vacuum and temperature (500° C.) the parts inclosed in the oven were sealed off from the pump and allowed to cool. Thereupon the charcoal tube, which was brought outside the oven, was placed into liquid air while the triangle, still within the oven, was again heated to 500° C. This temperature difference of nearly 700° C. was thus maintained for $5\frac{1}{2}$ hours. The charcoal tube was then sealed off from the triangle.

The extension micro-tip of the triangle (*m*, Fig. 1) was now placed deep into a four-liter beaker of triple-distilled water, there to remain while the water was boiled for about one hour. The tip was thereupon broken against the bottom of the beaker and the triangle was allowed to fill.

When the triangle had thus filled, the micro-tip with its small bulb was sealed instantly at the capillary constriction (Fig. 1) by means of an oxygen-gas micro-torch, the tip meantime, of course, remaining well below the surface of the water. We felt confident that this important seal was made air-tight but as an added precaution the sealed surface was quickly covered with an even layer of Dekhotinsky cement.

During the period of X-radiation, for $7\frac{1}{2}$ hours, which duly followed, the water within the triangle was constantly circulated by means of a heating coil (*h c.*) on one side and a cooling jacket (*c.j.*), through which ice water was passed, on another side (fig. 1).

Immediately following the irradiation, suitable portions of the water were removed from the triangle to be tested (1) for H_2O_2 and (2) for toxicity of Colpidia.

The capacity of the triangle was about 12 cc. so that a number of tests were possible for the H_2O_2 and for any toxicity on Colpidia which this oxygen-free, X-radiated water might show. The tests for H_2O_2, using again 1.0 $N TiCl_4$, were this time carried out with the aid of a Zeiss micro-comparator.

In all, 5 samples of water were taken from the triangle and subjected to tests for H_2O_2. Each of these 5 samples gave negative results. Our technique throughout had been most exacting and so the results were reckoned to be reliable. If so, then in the absence of oxygen in this X-radiated water, no hydrogen peroxide was formed, at least at any concentration susceptible of test with the $TiCl_4$. Comparing this technique with that of the previous experiment (1) where unbaked and unevacuated ampules of only one-twelfth the volume were used, the discrepancy in results is possibly accounted for.

Other samples of this X-radiated water were removed from the triangle and tested for any toxic action on Colpidia. Into each of 4 depression slides were placed 9 drops of the triple-distilled X-

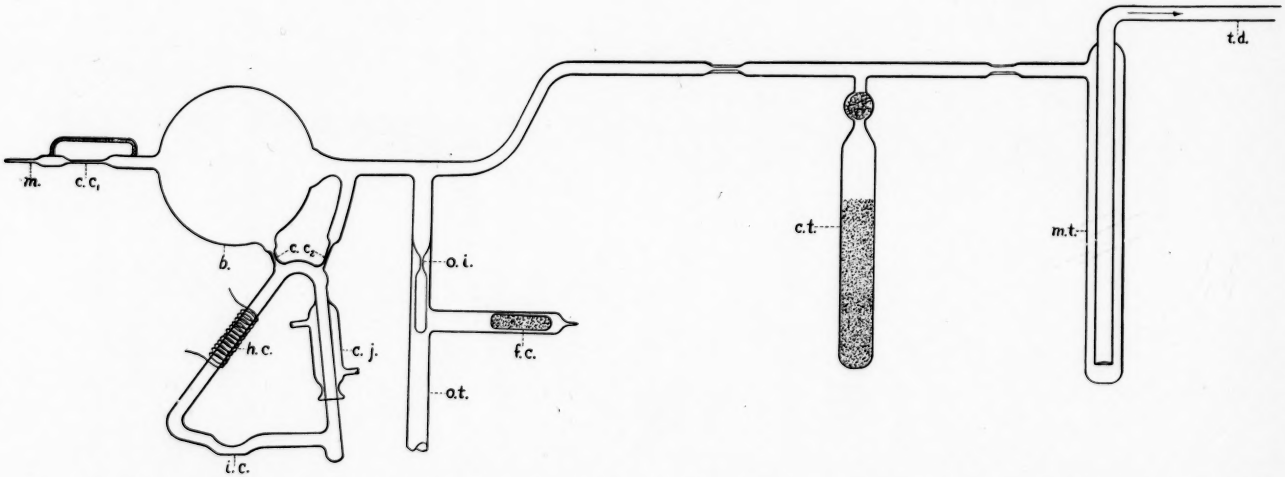

FIG. 1.—Triangle and accessories for obtaining oxygen-free water. *b*, bulb; *c.c*$_1$ and *c.c*$_2$, capillary constrictions; *c.j*, cooling jacket; *c.t*, charcoal tube; *f.c*, iron-fillings core; *h.c*, heating coil; *i.c*, thin surface for irradiation; *m*, micro-tip; *m.t*, mercury trap; *o.i*, capillary trap; *o.t*, tube to oxygen tank; *t.d*, tube to vacuum pumps.

radiated water and 1 drop of sterile balanced medium of 10X concentration. Before introducing the ciliates into the slides, they were washed 5 times in sterile balanced medium suitably diluted with unirradiated distilled water. The results were as shown in Table VI.

Also, a number of similarly washed Colpidia were introduced into a larger volume of the X-radiated water+balanced medium (prepared as above) contained in a watch crystal. None of these organisms showed any injurious effects.

Accordingly, from this biological criterion, also, there was no evidence of the presence of H_2O_2.

3. By means of the triangle apparatus, already described, we undertook further to determine the effect of X-radiating air-free

TABLE VI

Slide	Colpidia added	Results
1	5	No visible injury
2	5	No visible injury
3	10	No visible injury
4	10	No visible injury

water which had been saturated with oxygen at 20° C. and atmospheric pressure. Here again the triangle and accessory parts were baked for several days at 500° C. and evacuated approximately to 1×10^{-5} mm. Hg. At this pressure and temperature, seal-off was made and the glass parts within the oven, including the charcoal tube, were allowed to cool. As before, the sealing off of the charcoal tube was done after this had been placed in liquid air, while the other part of the apparatus was being heated at 500° C. for several hours.

The evacuated triangle and its attached bulb were then sealed air-tight with cement to an oxygen tank, through tube *o.t.* (Fig. 1), provided with a suitable mercury manometer which included a bulb of gold leaf for protection against Hg vapor. Thereupon, the triangle and affixed parts were partly filled with a given volume of triple-distilled water, which had been boiled for more than an hour, through the micro-tip (*m*, Fig. 1). When the water had reached a proper height, the tip and its bulb were sealed off quickly by means

of a micro-torch. The resulting sealed surface was at once covered with cement, although again we were quite certain that this surface had been made air-tight.

By means of the iron-filings core which is enclosed in the upright tube (*f.c.*, Fig. 1) continuous with the tube leading to the oxygen tank, the capillary trap (*o.i.*) was then broken. And so the water in the large bulb and triangle was exposed to oxygen from the tank. A compression valve on the tank regulated the oxygen pressure which was more accurately measured by the manometer.

With an extensive surface of water continuously thus exposed to oxygen at atmospheric pressure and 20° C., the water in the triangle was circulated through the bulb by keeping the lower tip of the triangle constantly in ice water. This procedure was carried on for more than four days in order to make reasonably certain that the contained water was completely saturated.

Thereafter, the triangle was successfully sealed off from the bulb and X-radiated for 7 hours. Throughout this period of X-radiation the water within the triangle was circulated as described for the former experiment (2).

Immediately following this exposure to the X-rays, the triangle was opened and samples of the irradiated water were removed for tests (1) for H_2O_2 and (2) for any toxic effects on Colpidia:

1. The tests for H_2O_2 were made as before with 1.0 $N$$TiCl_4$ with the aid of a Zeiss micro-comparator. The results were clear cut, such that it was possible not only to demonstrate the presence of hydrogen peroxide but also to approximate its concentration as compared with known dilutions of Merck's H_2O_2. From three independent estimates, it was evident that this concentration was near 1:90,000, and certainly greater than 1:100,000.

2. With other samples of this X-rayed water, its toxic action on Colpidia was also clearly shown.

H. THE TOXICITY OF HYDROGEN PEROXIDE TO *Colpidium campylum* FROM VIGOROUS CULTURES

As previously stated, comparator tests with titanic chloride for relative amounts of hydrogen peroxide in the X-radiated water, as compared with known dilutions of Merck's H_2O_2, showed concen-

trations of the former to be greater than 1:100,000. We had found such X-rayed water to be highly toxic to Colpidia.

As a further check on the estimated concentrations of the peroxide produced by irradiation, we undertook to determine in a series of accurately prepared dilutions of new, unopened Merck's stock, the minimal amount of H_2O_2 lethal to Colpidia taken from a vigorous culture. Scrupulously clean glassware was used throughout the series:

TABLE VII

Slide	Concentration of 1 Part H_2O_2 in H_2O_2	Observation 12 Hours Later
1	100	All dead. Granular remains visible
2	10,000	All dead. Granular remains visible
3	10,000	All dead. Granular remains visible
4	50,000	All dead. Granular remains visible
5	100,000	All dead. Granular remains visible
6	100,000	All dead. Granular remains visible
7	100,000	All dead. Granular remains visible
8	200,000	All dead. Granular remains visible
9	250,000	All dead. Granular remains visible
10	300,000	All dead. Granular remains visible
11	350,000	All dead. Granular remains visible
12	400,000	1 alive Slow. Granular remains of the Died within 2 hours others visible later.
13	450,000	All dead. Granular remains visible
14	500,000	All dead. Granular remains visible
15	500,000	1 alive. Rounded up. Granular remains of others Very sluggish move- visible ment

1. Of a thriving 4-day-old culture of *Colpidium campylum* in 10 per cent yeast extract, 3 cc. was diluted to a volume of 15 cc. with a balanced salt medium. The dilution was then centrifuged and its resulting supernatant portion decanted. This washing process was repeated twice. Thereupon, from 5 to 10 of the washed Colpidia were added to approximately 1/8 cc. portions of the following dilutions of Merck's H_2O_2, made up with balanced salt medium with results as shown in Table VII.

Twelve control cultures were made in which the animals all remained alive and active, though reduced in size due to the lack of food.

From the results listed in Table VII it is seen that all of the prepared dilutions of H_2O_2 down to the exceedingly low concentration of 1:500,000 were lethal to the Colpidia. The other conditions here imposed, including the absence of extraneous organic material, were fairly comparable with those obtaining for toxicity tests with the X-radiated water, recalling, however, that in the latter case a mea-

TABLE VIII

Slide	Final Concentration of H_2O_2	Observation 12 Hours Later
1	1 part in 200	All dead. Granular remains visible
2	1 part in 200	All dead. Granular remains visible
3	1 part in 200	All dead. Granular remains visible
4	1 part in 20,000	All dead. Granular remains visible
5	1 part in 20,000	All dead. Granular remains visible
6	1 part in 100,000	All dead. Granular remains visible
7	1 part in 100,000	Two alive. Granular remains of the 8 other *Colpidia* visible
8	1 part in 200,000	All alive. Sluggish in movement
9	1 part in 200,000	All alive. Sluggish in movement
10	1 part in 200,000	All alive. Sluggish in movement
11	1 part in 400,000	All alive. Sluggish in movement
12	1 part in 500,000	All alive. Sluggish in movement
13	1 part in 600,000	All alive. Sluggish in movement
14	1 part in 700,000	All alive. Sluggish in movement
15	1 part in 800,000	All alive. Sluggish in movement division of 4 individuals occurred
17	1 part in 900,000	All alive. Sluggish in movement
18	1 part in 1,000,000	All alive. Active
19	1 part in 1,000,000	All alive. Active
20	1 part in 1,000,000	All alive. Slower in movement than those of slides 18 and 19

ger amount of organic substance (yeast extract) was unavoidably introduced with the Colpidia.

It was decided, therefore, to run a second series with this organic material, yeast extract, added, noting any differential effects due to its presence.

2. To approximately 1/16 cc. portions of known dilutions of Merck's H_2O_2, made up, as before, with balanced salt medium, was added in each case 1/16 cc. of 10 per cent years extract. Then 5 to 10 Colpidia which had been washed three times, as in (1) above, were introduced into each slide. Controls were run. The results are shown in Table VIII.

After twelve hours, each of the 6 control slides showed thriving cultures of Colpidia.

Comparing the tabulated results of the 2 experimental series above, it is obvious that the presence of the yeast medium markedly reduced the toxic action of the hydrogen peroxide in the given dilutions of Merck's stock.

I. POSSIBLE EFFECTS ON THE YEAST MEDIUM FROM THE ADJACENT IONIZED AIR

As formerly mentioned, it might be assumed that the lethal state of the irradiated medium could be indirectly due to a diffusion into it of adjacent air ionized by the X-rays during the experimental exposure. It will be recalled that the yeast medium to be exposed to the rays was contained in amounts of only 0.05 cc. on the floor of the depression slide. The depression was thereupon sealed over by a very thin cover of mica. The resulting, relatively large volume of air thus confined might conceivably give rise, through the ensuing action of the X-rays, to toxic substances whose ready diffusion into the adjacent medium would, at least in part, account for the subsequent injury or death of the added organisms.

We performed no experiments in which the air alone was X-radiated since we had found that the irradiation of ampules containing only water and dissolved air yielded H_2O_2 in toxic doses comparable with those produced in our experimental slides. To extend this investigation a method much more efficient than that of X-ray absorption was employed to produce high-speed electrons in air. The chemical products from air affected by this method of the brush discharge or ozonizer, while possibly not identical with those of X-rays, are presumably comparable.

A volume of 5 cc. of the 10 per cent yeast medium was subjected to a continuous stream of ozonized oxygen which had passed through two washings of a solution of $KMnO_4$. Oxygen rather than air was thus treated since the electric discharge is known to affect almost exclusively the oxygen of the air thereby producing ozone (Mellor, 1922, p. 882). Colorimetric test with $TiCl_4$ failed to demonstrate any trace of H_2O_2 in this ozonized medium, and any other compound such as NH_3, NO, and NO_2 were presumably eliminated also by the $KMnO_4$ solution.

It was possible by this means to demonstrate that the ozonized medium, of sufficient concentration, was toxic to the Colpidia and that the medium itself could be bleached of its amber color by the ozone. A conspicuous difference was noted, however, in the appearance of the Colpidia killed by the ozonized medium and those killed by the X-rayed medium. In the former, the dead organisms were found at or near the surface of the medium and were not disintegrated. In X-rayed medium, on the contrary, the dead Colpidia, in the many cases observed, appeared on the bottom of the container and were disintegrated.

It would seem then that the lethal agent is not the same in these two sorts of treated yeast media.

J. THE PROTECTIVE ACTION OF VARIOUS ORGANIC MATERIALS AGAINST THE TOXICITY OF HYDROGEN PEROXIDE ON COLPIDIA

Evidence for a protective action of one organic material, the yeast extract, against the toxic effects of hydrogen peroxide on Colpidia was presented in the tabulated series of Section H above. Previous to this finding, however, we had sought to discover a protective agency in various organic substances, having surmised that hydrogen peroxide was an important toxic product in our X-radiated culture medium.

Aware of the readily decomposing action of catalase on H_2O_2, we tested the effect of sheep's blood on the toxicity of the irradiated medium as follows:

Into each of 11 depression slides was placed 1 drop (about 2.5×10^{-3} cc.) of defibrinated sheep's blood in 0.08 cc. of tap water. Three controls were run. Both experimental and control slides were given X-ray dosages of 20×10^4 to 360×10^4 R. Following the irradiation, 5 to 10 Colpidia were added with yeast medium (to a final concentration of 10 per cent). In each of the 11 experimental slides all of the Colpidia remained normal and produced good cultures. On the other hand, the Colpidia in the 3 control slides (which lacked the sheep's blood) all died.

In a further series of only 3 slides, the defibrinated sheep's blood, together with the years extract and the Colpidia, was added *after* the X-radiation of the slides containing tap water. The dosages of X-rays were 20×10^4 to 35×10^4 R. In 1 of the 3 lots the Colpidia

subsequently died while those in the remaining 2 slides were not perceptibly injured and they continued to reproduce.

The evidence, then, so far as it goes, indicates that the sheep's blood served, with 1 exception, to counteract the deleterious effect of X-radiated tap water on the Colpidia, whether the blood was added before or after the irradiation.

Comparable results were obtained upon using agar, gelatin, and bacteria as the protective organic materials. For example:

1. Three slides were prepared, such as noted above for the sheep's blood, adding an 0.08 per cent agar solution to 2 slides *before* the X-radiation and to the third slide *after* applying the X-rays. The dosages of X-rays were 34×10^4 to 45×10^4 R. The Colpidia, which

TABLE IX

Slide	X-Ray Dosages	Results
1	100×10^4	All lived, giving good cultures
2	200×10^4	All lived, giving good cultures
3	420×10^4	All lived, giving good cultures

with yeast extract were subsequently added, were not perceptibly affected in any of the 3 lots and continued to reproduce actively. In the controls, the organisms all died.

2. Two drops of a 1 per cent gelatin solution were added to 25 drops of tap water. With this dilution 3 depression slides were prepared and X-radiated at dosages of 34×10^4 to 80×10^4 R. units. The Colpidia, together with the yeast medium, were then added. No injurious effects, however, could be detected. For a fourth slide of the series, the dilute gelatin solution was introduced *after* the X-radiation (of equivalent dosage), along with the yeast medium and the ciliates. But similarly, the Colpidia showed no signs of injury. Controls were run and in these all the organisms died.

3. For the same purpose two species of bacteria were tried, *Bacillus coli* and *Pseudomonas fluorescens*. From a suspension of these in sterile tap water, 0.05 cc. portions were placed in 3 depression slides and, immediately following the X-radiation, Colpidia were added to each slide. The results are shown in Table IX.

The dosages of X-rays here were exceptionally high, yet the ciliates, subsequently added, were apparently completely protected by the bacteria from any toxic action of the X-radiated tap water.

DISCUSSION

The experiments which have been reported in foregoing pages were carried out as a consequence of our initial finding that the X-radiation of a sterile medium of 10 per cent yeast extract rendered that medium lethal to *Colpidium campylum* transferred from a vigorously growing bacteria-free culture.

We found further that the medium became also highly toxic or lethal to Colpidia when prepared from either tap or distilled water which had been X-rayed in dosages around 35×10^4 R. units.

Tests for the lethal agent or agents in the irradiated water disclosed the presence of hydrogen peroxide in concentrations certainly above 1:100,000. From a careful series of dilutions of Merck's 30 per cent H_2O_2, of new and unopened stock, it was demonstrated that concentrations greater than 1:450,000 were decidedly lethal to all the Colpidia introduced.

If the distilled or tap water to be X-rayed contained air, as through ordinary exposure, then the tests with the highly sensitive, specific reagent $TiCl_4$ (Ato, 1930; Barnaby and Isham, 1910, and Thornton, 1927) immediately following ample irradiation, were consistently positive. With the aid of micro-comparator tubes, the evidence was unmistakable.

The X-radiation of air-free, oxygenated water, at similar dosages, effected in it also the formation of hydrogen peroxide in concentrations that were readily detectable with the titanic chloride and were highly toxic or lethal to the Colpidia.

De-oxygenated water, on the other hand, if confined within glass containers that had been intensely baked and highly evacuated, did not contain, immediately following the dosages of X-rays, any hydrogen peroxide toxic to the Colpidia or susceptible of demonstration with the titanic chloride reagent. These latter results confirm the recent findings of Glocker and Risse (1928) and of Risse (1930, 1929*a*, 1929*b*) whose excellent studies were unknown to us during the progress of our experiments. From thorough and quite extensive in-

vestigations these workers reached the conclusion that hydrogen peroxide is not formed when water devoid of oxygen is X-radiated, and that for water containing oxygen, the amount of H_2O_2 produced by a given dosage of X-rays varies directly with the concentration of the dissolved oxygen.

Risse (1930) showed further that the decomposition of hydrogen peroxide could be greatly accelerated by X-rays. This decomposition was observed in concentrations as low as M/600. It would be expected, then, that at some point below this value an equilibrium mixture, in which the rate of formation was equal to the rate of decomposition, would result if the X-radiation were sufficient. In our experiments we multiplied many times the dose required to produce detectable quantities of hydrogen peroxide in undertaking to increase the concentration. But it was found that the concentration did not increase proportionally. It seems likely, therefore, that at least in many cases we had equilibrium mixtures in which the concentration of hydrogen peroxide depended only on the concentration of the dissolved oxygen. Our concentrations of H_2O_2 were comparable with those obtained in work cited by Risse (1930, p. 285), viz., M/10,000.

Tests with various organic materials including sheep's blood, agar, gelatin, and bacteria, when added to tap water either before or after its exposure to the X-rays, showed that such materials were protective against the toxic action of the irradiated water on Colpidia. It was found also that in carefully prepared dilutions of Merck's H_2O_2 the addition of yeast extract definitely diminished the toxicity of the peroxide to the washed Colpidia.

Assuming that the deleterious effect of the X-radiated medium or water was in large measure due to the production there of hydrogen peroxide, it appears quite probable that the detoxification by the several organic materials involved essentially their decomposing action on the H_2O_2. This interpretation is made provisionally, however, since our data as yet remain inadequate. Moreover, it has been found by others (Brown, Luck, Sheets, and Taylor, 1933), working in this laboratory, that beef broth or yeast extract did not, when X-radiated, prove toxic to either of 2 species of bacteria (the same as used in our experiments) *Pseudomonas fluorescens* and

Bacillus coli, and that the X-radiation of a culture medium containing as food these same bacteria did not render that medium toxic to the culture ciliate, *Euplotes taylori*. This discrepancy may be due to the inherent differences between the organisms involved, such as the presence or absence of a protecting catalase, or possibly to some unknown reactions within the X-radiated organic medium. Much more work is required to elucidate this and other highly interesting phases of our general problem.

It should here be emphasized that the X-ray production of other toxic agents in the yeast medium appears not improbable, so that we can at present regard hydrogen peroxide as only 1 of several possible toxic agents thus producible. It is hoped that investigations now under way may yield a more complete analysis of the induced factors contributory to this lethal condition.

The extreme toxicity of hydrogen peroxide, however, together with its demonstrated frequency, concentration, and source in this X-radiated medium strengthens our present assumption that its rôle here is one of major importance.

SUMMARY

1. The X-radiation of a sterile medium of 10 per cent yeast extract rendered the medium lethal to *Colpidium campylum*.
2. X-radiated tap or distilled water was found also highly toxic or lethal to the Colpidia.
3. Tests with titanic chloride disclosed hydrogen peroxide in the irradiated water, in concentrations above 1:100,000.
4. Concentrations of Merck's H_2O_2, new stock, above 1:450,000 were lethal to Colpidia.
5. Air-free, oxygenated water, when X-radiated, gave positive tests for H_2O_2 and proved toxic to Colpidia.
6. De-oxygenated water, following the irradiation, gave negative tests for H_2O_2 and was not toxic to Colpidia.
7. Products from an ozonizer, presumably similar to those from X-ray absorption by air, proved toxic to Colpidia but in a manner different from that of X-rayed medium.
8. Organic materials, including sheep's blood, agar, gelatin, and bacteria, added to tap water before or after its X-radiation, protected Colpidia against its toxic action.

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THE EFFECT OF ALTERNATING TEMPERATURES ON THE PUPAL DEVELOPMENT OF DROSOPHILA MELANOGASTER MEIGEN

(One figure)

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THE generalization has frequently been made that varying temperatures have a stimulating effect on development as compared with their mean constant temperature. Shelford (1927) found that variable, as compared with constant temperatures, accelerated all developmental stages of the codling moth. Proportional increases were as noted as follows: eggs, 7 per cent; larvae, 8 per cent; and pupae, 7 per cent. Cook (1927) found that when first instar larvae of the cutworm, *Porosagrotis orthogonia*, were alternated between 8° C. and a high temperature an acceleration of the development resulted. Peairs (1927) observed a definite acceleration in the development of blow fly larvae exposed to temperatures changed at 6–12 hour intervals. Parker (1930) showed that in the nymphs of the grasshopper, *Melanoplus mexicanus*, alternating between 22° C. and a higher temperature such as 27° caused an acceleration of 12.4 per cent. When 12° was used as the low temperature, the acceleration was 45.8 per cent.

A few experiments, however, tend to show that varying temperatures may also produce a retardation of development. Headlee (1914) studied the development of the grain aphis, *Toxoptera graminum*, and found that a constant temperature was more effective in development than a fluctuating temperature with the same mean. In 1929 he obtained similar results with the codling moth, *Carpocapsa pomonella*.

Ludwig (1928) suggested that, in the Japanese beetle, the effect produced by alternating temperatures may depend upon the temperatures involved.

1. If one of the temperatures is above the optimum of development and the other is between the threshold and the optimum, the rate is retarded. He believed that this may be explained by the assumption that, while the organisms are exposed to temperatures above the optimum, their development is retarded to such a degree that the total developmental time is greater than at the corresponding constant temperature.

2. If both of the temperatures used are between the threshold and the optimum of development, neither an acceleration nor a retardation of rate is produced.

3. If one of the temperatures involved is below the threshold of development and the other is between the threshold and the optimum, the rate is accelerated.

The present study is an attempt to test the validity of these postulates by determining the effect of daily alternations between two temperatures on the duration of the pupal stage of *Drosophila melanogaster*.

MATERIAL AND METHODS

The stock of *Drosophila* used in these experiments was secured from Dr. C. R. Plunkett of New York University, and had been inbred for several years. The standard banana-agar culture medium was employed. The cultures were kept in an incubator at approximately 25° C., and development up to the pupal stage was allowed to take place at that temperature. To facilitate the transference of pupae, the flies were cultured in one-half pint mayonnaise jars. Since no filter paper was added, the larvae pupated on the sides of the jar and could be removed very readily. Pupae were collected every hour, so that, when obtained, they were always within the first hour after pupation. When the larvae became quiescent and protruded the anterior horns, they were identified as pupae. Each pupa was placed in a separate test tube, in the bottom of which was a wad of moist cotton. The tubes were then stoppered with cotton, labeled, and placed in a water bath at the proper temperature. Temperatures were controlled to within 0.1 of a degree C. Temperatures below that of the room were secured by running tap water through a coil of copper tubing placed in the water bath. When lower temperatures were desired, the water was cooled by first pass-

ing it through a coil placed in an ice bath. When there was any reason to doubt the accuracy of water-bath temperature, the experiment was repeated. Alternations between temperatures were made every twenty-four hours, and a record was kept of the length of time each pupa was exposed to the high and low temperature. Readings on emergence were also made every hour, except at 15° and at alternating temperatures of 10° and 20°, when they were made at eight-hour intervals.

TABLE I

DURATION OF THE PUPAL STAGE OF DROSOPHILA AT CONSTANT TEMPERATURES

Males					Females				
Temperature (Degrees C.)	No. Flies Emerged	Percentage Emerged	Time in Days	Percentage Development per Day	Temperature (Degrees C.)	No. Flies Emerged	Percentage Emerged	Time in Days	Percentage Development per Day
10	0	0	.....	.....	10	0	0	.....	.....
15	40	96.4	14.15±0.036	7.06	15	42	96.4	13.54±0.022	7.38
20	92	96.7	6.78±0.008	14.74	20	82	96.7	6.45±0.006	15.29
22.5	63	98.6	5.27±0.012	18.97	22.5	70	98.6	4.93±0.007	20.28
25	118	98.2	4.26±0.007	23.47	25	100	98.2	4.02±0.004	24.87
26	35	100.0	4.00±0.006	25.00	26	42	100.0	3.81±0.004	26.24
27	108	97.6	3.78±0.003	26.45	27	101	97.6	3.59±0.004	27.85
27.5	110	99.5	3.65±0.002	27.39	27.5	124	99.5	3.48±0.004	28.73
28	71	95.9	3.56±0.005	28.09	28	94	95.9	3.37±0.003	29.67
28.5	47	92.6	3.45±0.004	28.98	28.5	53	92.6	3.27±0.004	30.58
29	104	99.5	3.40±0.004	29.41	29	102	99.5	3.22±0.003	31.05
29.5	105	99.9	3.35±0.004	29.85	29.5	110	99.9	3.17±0.003	31.54
30	66	95.6	3.35±0.003	29.85	30	63	95.6	3.23±0.004	30.96
31	21	92.7	3.35±0.008	29.85	31	17	92.7	3.21±0.014	31.15
32	24	78.2	3.50±0.008	28.57	32	24	78.2	3.36±0.010	29.76
33	17	38.7	3.63±0.016	27.57	33	7	38.7	3.50±0.017	28.57
34	0	0	.....	.....	34	0	0	.....	.....

To determine the rate of development at each temperature, a series of constant temperature experiments was performed. In every case, records were kept of the duration of the pupal stage and the sex of each adult which emerged.

OBSERVATIONS

Effects of constant temperatures.—The results obtained at constant temperatures are shown in Table I and Figure 1. At 10° C., which is below the theoretical threshold for pupal development (the point at which the rate curve crosses the temperature axis), no flies emerged. They appeared to be completely formed, however, within the pupal cases after 40–45 days, showing that development occurred at 10°.

At 34° no flies emerged, although in a few cases they also appeared to be completely formed within the pupal cases. Table I shows that the pupal stage is of longer duration in males than in females. This is in agreement with the work of Bonnier (1926). Between 27° and 30°, readings were made at one-half degree intervals to determine the

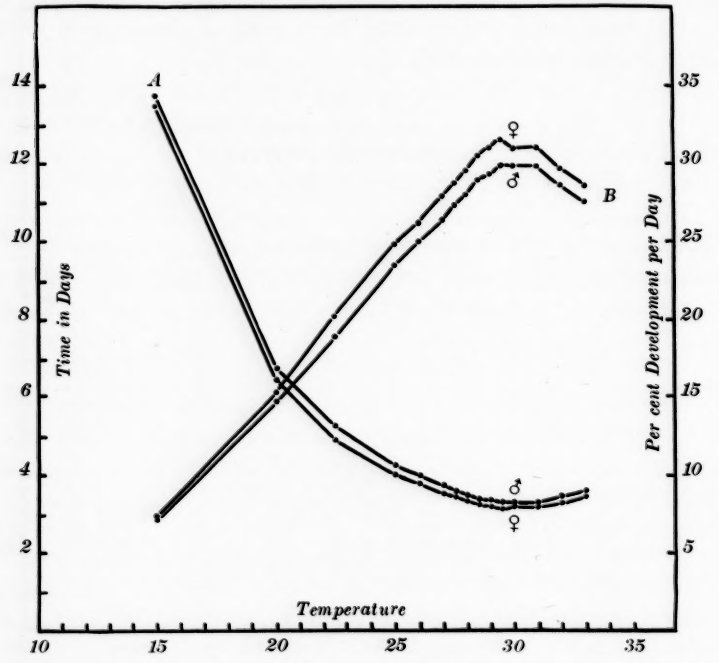

FIG. 1.—Effect of constant temperatures on pupal development. *A*, time curves; *B*, rate curves.

temperature at which development occurs most rapidly. This was found to be 29.5° for both sexes. Table I also contains the percentages of development per day which occur at each constant temperature. These figures are used in evaluating the effects of alternating temperatures.

Effect of alternating temperatures, one of which is above the optimum.—Table II gives the results of daily alternations between two tem-

peratures when one is above the optimum for pupal development. A comparison of the values given in this table with those in Table I (for constant temperatures) will show that in every case there is a retardation of development as compared with the rate at the temperature corresponding to their mean. For example, when placed first at 24° and alternated with 33°, the duration of the pupal stage in the female average 3.88 ± 0.014 days, while at 28.5°, the mean of 33 and 24, it averaged only 3.27 ± 0.004 days. When alternated between 24° and 33° but exposed first to 33°, the time was increased to 4.17 ± 0.019 days. A possible exception to this statement occurred when the pupae were alternated daily between 25° and 30°. When exposed to 25° the first day, it required 3.50 ± 0.004 days for the development of the female pupae as compared with 3.48 ± 0.004 days at 27.5°. However, in this case the pupae were exposed to 30° for 2.04 days and to 25° for only 1.46 days.

The suggestion was made by Ludwig (1928) that this retardation may be due to the slower developmental rate at constant temperatures above the optimum. To test this hypothesis, the percentage of development which occurs at each temperature was calculated by multiplying the time of exposure (in days) by the percentage of development per day at the respective temperatures. By adding the percentages of development at the temperatures used, approximately 100 per cent should be obtained if the hypothesis is a satisfactory explanation. Table II shows that values of approximately 100 are obtained when the pupae are exposed to the low temperature the first day after pupation. When they are exposed to the high temperature first, however, and when a temperature as high as 32° or 33° is used, a definite retardation above that expected on the basis of constant temperature experiments occurs in both sexes. It is interesting to note (Table I) that the percentage of emergence resulting from constant exposure to these temperatures is also lowered. This observation suggested that perhaps the entire pupal stage is not equally susceptible to the retarding influence of exposure to high temperatures.

To determine whether there are differences in susceptibility during the course of pupal development, *Drosophila* pupae were collected and divided into four series. Series I was exposed to 33° during the

first 24 hours and then to 25° until the flies emerged. Series II was exposed to 25° for the first 24 hours, to 33° the second 24 hours, and finally to 25° until emergence. Series III was exposed to 25° for the first 48 hours, to 33° for the next 24 hours, and finally to 25° until emergence. Series IV was exposed to 25° for the first 72 hours, then

TABLE II

EFFECT OF ALTERNATING TEMPERATURES ONE OF WHICH IS ABOVE THE OPTIMUM

Temp.* (Degrees C.)	No. Flies Emerged	Time in Days			Percentage of Development				
					Per Day				
		Low Temp.	High Temp.	Total	Low Temp.	High Temp.	Low Temp.	High Temp.	Total
Males									
24 and 33	19	2.10	1.96	4.06±0.016	21.58	27.54	45.31	53.97	99.28
33 and 24	28	1.96	2.36	4.32±0.008	21.58	27.54	42.29	64.99	107.28
24 and 32	40	2.01	1.96	3.97±0.007	21.58	28.54	43.37	55.93	99.30
32 and 24	29	1.96	2.26	4.22±0.007	21.58	28.54	42.29	64.50	106.79
26 and 31	29	2.02	1.62	3.64±0.007	25.00	29.85	50.50	48.35	98.85
31 and 26	29	1.62	2.13	3.75±0.008	25.00	29.85	40.50	63.58	104.08
25 and 30	33	1.46	2.21	3.67±0.006	23.47	29.85	34.26	65.96	100.22
30 and 25	37	2.16	1.79	3.95±0.007	23.47	29.85	50.69	53.43	104.12
20 and 30	36	2.86	2.00	4.86±0.019	14.75	29.85	42.18	59.70	101.88
30 and 20	45	2.00	2.57	4.57±0.011	14.75	29.85	29.50	76.71	106.21
Females									
24 and 33	36	1.92	1.96	3.88±0.014	22.74	28.57	43.66	55.99	99.65
33 and 24	36	1.96	2.21	4.17±0.019	22.74	28.57	44.30	63.13	107.43
24 and 32	34	1.88	1.96	3.84±0.009	22.74	29.76	42.75	58.32	101.07
32 and 24	22	1.96	2.01	3.97±0.029	22.74	29.76	44.30	59.81	104.11
26 and 31	40	1.92	1.62	3.54±0.005	26.24	31.15	50.38	50.46	100.84
31 and 26	32	1.62	1.83	3.45±0.016	26.24	31.15	42.50	57.00	99.50
25 and 30	42	1.46	2.04	3.50±0.004	24.87	30.96	36.31	63.15	99.46
30 and 25	39	2.16	1.54	3.70±0.014	24.87	30.96	53.71	47.67	101.38
20 and 30	25	2.50	2.00	4.50±0.026	15.50	30.96	38.75	61.92	100.67
30 and 20	31	2.00	2.34	4.34±0.009	15.50	30.96	31.00	72.44	103.44

* In the tables of alternating temperature experiments, the manner of exposure is indicated by the order in which the temperatures are given. For example, if the temperatures are stated as 24° and 33°, the pupae were exposed the first 24 hours to the 24° and the second 24 hours to 33°.

to 33° until emergence. The results of this experiment are given in Table III. In both sexes a decided retardation of development is produced when the pupae are exposed to 33° during the first 24 hours of pupal development. When the exposure to 33° occurred later in pupal development, however, the results indicated a sex difference. The males are retarded during the second-day exposure and accelerated during the fourth-day exposure to 33°. This acceleration dur-

ing the fourth day is sufficient to cancel the retardation resulting from exposure to 33° during the second day. In an alternating temperature experiment between 25° and 33°, when the pupae are placed at 33° the first day and transferred at 24-hour intervals, theoretically there should be a retardation of nearly 7 per cent. Table II shows that a retardation of 7.28 per cent did occur with male pupae in a similar experiment. Exposure of female pupae to 33° on the second,

TABLE III

EFFECT OF A SINGLE 24-HOUR EXPOSURE TO 33 DEGREES C. DURING DIFFERENT PERIODS OF PUPAL DEVELOPMENT, REMAINDER OF TIME AT 25 DEGREES C.

Period at 33 Degrees	No. Flies Emerged	Time in Days			Percentage of Development					
					Per Day					
		Low Temp.	High Temp.	Total	Low Temp.	High Temp.	Low Temp.	High Temp.	Total	Total −100
Males										
First day...	61	3.31	1.00	4.31±0.009	23.47	27.54	77.68	27.54	105.22	+5.22
Second day.	64	3.27	1.00	4.27±0.009	23.47	27.54	76.74	27.54	104.28	+4.28
Third day..	62	3.14	1.00	4.14±0.020	23.47	27.54	73.69	27.54	101.23	+1.23
Fourth day.	66	3.00	0.92	3.92±0.005	23.47	27.54	70.41	25.33	95.74	−4.26
Females										
First day...	39	3.08	1.00	4.08±0.009	24.87	28.57	76.59	28.57	105.16	+5.16
Second day.	53	2.94	1.00	3.94±0.006	24.87	28.57	73.11	28.57	101.68	+1.68
Third day..	58	2.82	1.00	3.82±0.011	24.87	28.57	70.13	28.57	98.70	−1.30
Fourth day.	51	3.00	0.86	3.86±0.007	24.87	28.57	74.64	24.57	99.21	−0.79

third, or fourth days resulted in no appreciable change in the rate of development. In Series IV, this acceleration of male pupae was great enough to eliminate the sex difference in the duration of the pupal stage, male and female flies emerging together.

This experiment was repeated and similar results were obtained a second time.

Effect of alternating temperatures when both are between the theoretical threshold and the optimum.—Table IV gives the results of alternating temperature experiments when each temperature is between the optimum and the theoretical threshold of development. If the total percentage of development is less than 100, an acceleration

occurs; if it is greater, there is a retardation of development. With the exception of one experiment, when pupae were alternated between 21° and 29° (exposed to 29° the first day), there was neither an acceleration nor a retardation of development. This exception might possibly be explained by the fact that the values used in calculating the percentage of development per day at 21° were not obtained by an experiment at 21°, but were calculated from the rate curve based on experiments at other temperatures.

TABLE IV

EFFECT OF ALTERNATING TEMPERATURES BOTH OF WHICH ARE BETWEEN THE THEORETICAL THRESHOLD AND THE OPTIMUM

Temp. (Degrees C.)	No. Flies Emerged	Time in Days			Percentage of Development				
					Per Day				
		Low Temp.	High Temp.	Total	Low Temp.	High Temp.	Low Temp.	High Temp.	Total
Males									
21 and 29	35	2.52	2.00	4.52±0.007	16.72	29.41	42.13	58.82	100.95
29 and 21	30	2.00	2.39	4.39±0.004	16.72	29.41	33.44	70.28	103.72
22.5 and 27.5	42	2.31	2.00	4.31±0.010	18.97	27.39	43.82	54.78	98.60
27.5 and 22.5	32	2.00	2.17	4.17±0.012	18.97	27.39	37.94	59.43	97.37
15 and 25	28	3.95	3.00	6.95±0.007	7.06	23.47	27.88	70.41	98.29
Females									
21 and 29	35	2.17	2.00	4.17±0.007	17.61	31.05	38.31	62.10	100.41
29 and 21	27	2.00	2.23	4.23±0.014	17.61	31.05	35.22	69.24	104.46
22.5 and 27.5	29	2.01	2.00	4.01±0.008	20.28	28.73	40.76	57.46	98.22
27.5 and 22.5	26	1.97	2.00	3.97±0.008	20.28	28.73	39.95	57.46	97.41
15 and 25	40	3.38	3.00	6.38±0.016	7.38	24.87	24.94	74.61	99.55

Effect of alternating temperatures, one of which is below the theoretical threshold.—Table V contains the results of alternating temperatures on pupal development when one of the temperatures is below the theoretical threshold. When alternated between 10° and 20° (placed first at 10°) female flies emerged in an average of 11.59±0.030 days, while the average time required at 15° (Table I) was 13.54±0.022 days, and at 20° it was 6.45±0.006 days. If no development occurred at 10° it should have required twice the time necessary at 20°, hence, daily alternations between these temperatures gave an

acceleration of from 11 to 14 per cent in female, and 10 to 11 per cent in male, pupae. If it is assumed that development at 10° is completed in 42.5 days, an approximation derived from constant temperature experiments, no acceleration at alternating temperatures of 10° and 20° is obtained.

TABLE V

EFFECT OF ALTERNATING TEMPERATURES ONE OF WHICH IS BELOW THE THEORETICAL THRESHOLD

Temp. (Degrees C.)	No. Flies Emerged	Percentage Emerged	Time in Days		Percentage of Development				
					Per Day				
			Low Temp.	High Temp.	Low Temp.	High Temp.	Low Temp.	High Temp.	Total
Males									
7 and 27	13	40.6	.....	3.75±0.006	.....	26.45	.....	99.18	.....
27 and 7	27	96.8	.....	3.85±0.004	.....	26.45	.....	101.83	.....
8 and 27	21	59.2	.....	3.71±0.002	.....	26.45	.....	98.12	.....
27 and 8	38	100.0	.....	3.60±0.004	.....	26.45	.....	95.22	.....
9 and 27	33	78.2	.....	3.67±0.003	.....	26.45	.....	97.07	.....
27 and 9	36	97.3	.....	3.59±0.007	.....	26.45	.....	94.95	.....
10 and 27	55	78.3	3.62	3.56±0.011	2.38	26.45	8.61	93.63	102.24
27 and 10	67	97.3	3.39	3.73±0.017	2.38	26.45	8.06	96.65	104.71
10 and 20	12	90.2	6.66	6.10±0.040	2.38	14.74	15.85	89.91	105.76
20 and 10	20		5.77	6.04±0.021	2.38	14.74	13.73	89.02	102.75
20 and 10	30	76.0	5.22	6.07±0.034	2.38	14.74	12.42	89.47	101.89
Females									
7 and 27	13	40.6	.....	3.64±0.014	.....	27.85	.....	101.37	.....
27 and 7	25	96.8	.....	3.73±0.004	.....	27.85	.....	103.88	.....
8 and 27	22	59.2	.....	3.68±0.003	.....	27.85	.....	102.48	.....
27 and 8	35	100.0	.....	3.43±0.007	.....	27.85	.....	95.50	.....
9 and 27	26	78.2	.....	3.43±0.006	.....	27.85	.....	95.50	.....
27 and 9	36	97.3	.....	3.43±0.007	.....	27.85	.....	95.50	.....
10 and 27	55	78.3	3.61	3.34±0.013	2.38	27.85	8.59	92.99	101.58
27 and 10	57	97.3	3.41	3.37±0.007	2.38	27.85	8.11	93.83	101.94
10 and 20	15	90.2	6.00	5.59±0.030	2.38	15.50	14.28	86.64	100.92
20 and 10	18		5.03	5.75±0.036	2.38	15.50	11.94	89.12	101.06
20 and 10	24	76.0	5.00	5.59±0.009	2.38	15.50	11.90	86.64	98.54

Since by a constant temperature experiment at 10°, and by alternating between 10° and 20°, it was found that 10° is above the threshold of development, it was decided to place pupae at different low temperatures alternated with the same high temperature to determine, if possible, the actual threshold of development. In calculating the results of these experiments only the time at high temperatures was used to obtain the total percentage of development. If this value is 100 or above, it is assumed that no development occurs at

the low temperature. When pupae were alternated between 7° and 27°, the time spent at the high temperature was the same as the duration of the pupal stage at a constant temperature of 27°. This indicates that all of the development occurred at 27° and that 7° is below the threshold of pupal development. However, when pupae were alternated between 8° and 27° (exposed to 27° first), assuming that no development occurs at 8°, a definite acceleration of nearly 5 per cent occurs in both sexes. This observation indicates that the developmental threshold is between 7° and 8° and that some development does occur at 8°. These experiments further indicate that, in *Drosophila*, the development which occurs between the actual and the theoretical threshold of development is sufficient to account for the apparent acceleration of development when pupae are alternated daily between two temperatures, one of which is below the theoretical threshold of development.

The results in Table V also suggest that in different stages of pupal development there may be a differential susceptibility to low temperatures. When pupae were exposed to 7° the first day, only 40.6 per cent emerged, while when exposed to 27° the first day 96.8 per cent emerged. To determine definitely whether the stage of pupal development determines the ability of pupae to survive after exposure to low temperatures, a number of pupae were collected and divided into three series. Series I, containing 68 pupae, were placed at 10° the first day and then exposed to 27° until the adults emerged. Only 85 per cent emerged. Series II, containing 61 pupae, were placed at 27° the first day, 10° the second day, and then exposed to 27° until emergence. In this series 98.4 per cent emerged. Series III, containing 56 pupae, were exposed to 27° for two days, then to 10° for one day, and then returned to 27° and kept there until emergence. In this series 96.4 per cent emerged. These experiments demonstrate that during the first 24 hours following pupation there is a greater susceptibility to low temperature than during the remainder of the pupal stage.

DISCUSSION[1]

These results, obtained on the pupal stage of *Drosophila melanogaster*, are in agreement with those reported by Ludwig (1928) on

[1] Comprehensive discussions of the effects of constant and varying temperatures on insect development are found in the works of Shelford (1929), Uvarov (1931), and Chapman (1931), to which the reader is referred.

the egg, larval, and pupal stages of the Japanese beetle, *Popillia japonica*. They suggest a possible explanation for some of the apparently contradictory results obtained by many other investigators. It seems quite evident that most of the retarding effects obtained by alternating pupae between two temperatures, when one is above the optimum, are due to the fact that the rate curve at high temperatures no longer follows the straight-line relationship which obtains over the more favorable temperature range. Moreover, at constant temperatures as high as 32° and 33°, the percentage of flies emerging is much reduced, being 78.2 at 32° and only 38.7 at 33°. When alternating temperature experiments were performed involving these temperatures, it was found that the results depended upon the stage of pupal development when exposed to the high temperature. When exposed to 32° or 33° during the first 24 hours of pupal life, the retardation was greater than expected on the basis of constant temperature experiments. This suggests the development in older pupae of a resistance to the retarding effects of exposure to high temperature. Furthermore, a greater mortality was observed when pupae were exposed to a low temperature during the first 24 hours, also suggesting an increase in resistance with age. These results agree with those of Mavor (1927) who described differences in susceptibility of different stages of *Drosophila melanogaster* to X-rays. He found the egg, larva, and early pupa relatively susceptible to X-ray, resistance increasing about 20 hours after pupation. It then increases with great rapidity until the end of the pupal stage. At this time the dose required to produce 50 per cent mortality in 5 days after exposure was 35 times that required at the beginning of the pupal period. Mavor states that this increase in resistance coincides with the later part of metamorphosis after the main organs of the imago had been formed but are still undergoing differentiation.

The retarding effect of a single 24-hour exposure to a high temperature (such as 33°) on the rate of subsequent development at a lower temperature is in agreement with the results of Janisch (1930) on the moth, *Prodenia littoralis*. He found that short exposures to a high temperature retard the subsequent development at the optimum.

Many of the investigations dealing with the effects of varying temperatures on insect development have been made under field conditions where the temperature frequently goes below the thresh-

old of development. This may account for the opinion that varying temperatures accelerate insect development. Cook (1927), in his work on the cutworm, *Porosagrotis orthogonia*, used 8° alternated with different high temperatures. The accelerating effects reported by him may possibly be due to a greater rate of development at 8° than he supposed, since he performed no control experiment at that temperature but calculated the rate of development from his experiments at higher temperatures. The experiments reported in this paper for *Drosophila* pupae suggest that any acceleration produced when pupae are alternated between a temperature below the theoretical threshold and a high temperature is due to a certain amount of development which occurs at the low temperature. No experiments were performed using temperatures between 10° and 15°, but it is possible that accelerations might be produced, especially at the lower temperatures. Krogh (1914) found that in the meal worm, *Tenebrio molitor*, at low temperatures the observed results deviate from the straight line, development occurring more rapidly than is calculated on the basis of experiments conducted at higher temperatures. If one of these is used as the low temperature in an alternating temperature experiment, development would probably be accelerated as compared with the mean constant temperature.

The threshold of development has been defined by Peairs (1927) as the temperature at which, on the descending scale, the development definitely ceases, and at which, on the ascending scale, development is initiated. The results of Krogh (1914), Peairs (1927), and Shelford (1927) indicate that the actual threshold is below the theoretical threshold. Uvarov (1931) noted that "the practical difficulty of determining the exact threshold of development for a given stage of an insect is evidently very great, since it is scarcely possible to register by any method a very small amount of development going on at a very slow rate." Shelford (1929) states that few or no threshold points have been definitely determined but that they have been approximated. He further states that in all cases the use of these approximations have led to erroneous results. One method of calculating the developmental threshold, introduced by Oettingen (1879), is based on the concept of a thermal constant for development. If the time in days required for the completion of a given

stage of development is multiplied by the effective temperature, a constant value, the thermal constant, is obtained. This is expressed in day-degrees required for development. The effective temperatures for the stage are obtained by subtracting the threshold temperature from the temperature at which the animals were kept. Oettingen's method consists of making a series of observations on the duration of the developmental period at different constant temperatures. Then several probable values are assumed for the threshold tem-

TABLE VI

DETERMINATION OF THE THRESHOLD TEMPERATURE BY THE METHOD OF OETTINGEN

Temperature (Degrees)	Time in Days	Assumed Threshold Values			
		8°	9°	10°	11°
		Day Degrees	Day Degrees	Day Degrees	Day Degrees
15	13.79	96.53	82.74	68.95	55.16
20	6.78	81.36	74.58	67.80	61.02
22.5	5.27	76.41	71.14	65.87	60.80
25	4.26	72.42	68.16	63.90	59.61
26	4.00	72.00	68.00	64.00	60.00
27	3.78	71.82	68.04	64.26	60.48
28	3.56	71.20	67.64	64.08	60.52
29	3.40	71.40	68.00	64.60	61.20
29.5	3.35	73.03	68.67	65.32	61.97

perature, and thermal constants are calculated. The value which gives the most constant results over the widest range of temperatures is accepted as the threshold.

This method of obtaining the threshold of development was applied to the results obtained on *Drosophila* pupae at constant temperatures. In Table VI the day-degrees are given for pupal development using temperatures ranging from 8° to 11° as the threshold. This table shows that the most constant values are obtained when 10° is used, although both alternating and constant temperature experiments demonstrated that development does take place at this temperature. Therefore, it must be concluded that the method of Oettingen does not give the correct value for the threshold temperature. The data reported in this paper have indicated that alternat-

ing between different low temperatures and a high temperature is a satisfactory experimental method of determining the developmental threshold. In an alternating temperature experiment, if the animals must be exposed to the high temperature the same length of time for the completion of a given stage as is required in a constant exposure to the same temperature, it is obvious that no development occurs at the low temperature. However, if the time at the high temperature is less than is required in a constant exposure to the same temperature, some development occurs at the low temperature which, therefore, is above the developmental threshold.

Peairs (1927) attempted to find the actual threshold of development of blow-fly pupae by placing different groups in low temperature incubators for periods varying from 1 month to 2 years, after which they were kept at higher temperatures until the completion of development. He thought that if any measurable development had occurred while the pupae were exposed to the low temperature, it should be indicated by a corresponding reduction of the time necessary to complete development at the higher temperature. Actually, development was retarded after such prolonged exposure to low temperature, so that any development which may have taken place at the low temperature was not observed. A retardation of developmental rate is not apparent in 24-hour exposures to low temperatures.

These results have no application to experiments on the diapause eggs of grasshoppers. Bodine (1925) on *Melanoplus differentialis* and Parker (1930) on *Melanoplus mexicanus* found that if eggs were allowed to develop for some time at a favorable temperature and then exposed to a low temperature, subsequent development at the high temperature was greatly accelerated. Parker (1930) found that exposure to 0° resulted in a greater acceleration than an exposure to 8°. Bodine (1932) explains the action of cold on the diapause eggs of *Melanoplus differentialis* as follows: "Diapause eggs contain factors (X) which cause diapause in rather definite fashion and at definite stages in the eggs' development. Diapause factors (X) are extremely susceptible to low temperatures (below developmental zero) and can be completely destroyed or inhibited by appropriate exposure to these temperatures."

Hase (1927) with the eggs of the flour moth, *Ephestia kuehniella*,

and (1930) with the eggs of the bedbugs, *Cimex lectularius* and *Cimex rotundatus;* Janisch (1930) with the eggs of the moth, *Prodenia littoralis;* and Ludwig (1930) with the eggs of the Japanese beetle, *Popillia japonica*, found that exposure to low temperature delays the time of hatching at the optimum temperature as compared with eggs kept at a constant optimum temperature. In these cases, however, the eggs were not alternated between a low and a high temperature as in the present experiments.

SUMMARY

1. The results of exposing *Drosophila* pupae to daily alternations between two temperatures depend upon the temperatures involved. (*a*) If one of the temperatures is above the optimum of development and the other is between the theretical threshold and the optimum, development appears to be retarded. This retardation may be due to the reduced rate of development at temperatures above the optimum so that the total developmental time is longer than at the mean constant temperature. (*b*) If both temperatures are between the theoretical threshold and the optimum, neither an acceleration nor a retardation is produced. (*c*) If one temperature is between the theoretical threshold and the actual threshold of development, the rate is accelerated. This acceleration is due to development which occurs at temperatures below the theoretical threshold.

2. *Drosophila* is more susceptible to the retarding influence of exposure to high temperatures during the first 24 hours of pupal life than in the remainder of this period. Exposure to low temperatures during the first 24 hours of pupal life also results in a higher mortality than a similar exposure later in pupal development.

3. Alternating *Drosophila* pupae between different low temperatures and a high temperature is a satisfactory method of determining the developmental threshold. In an experiment of this nature, if the pupae must be exposed to the high temperature the same length of time for the completion of a given stage as is required in a constant exposure to the same temperature, no development occurs at the low temperature. However, if the time at the high temperature is less than is required in a constant exposure to the same temperature, some development occurs at the low temperature which, therefore, is above the developmental threshold.

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THE RELATION BETWEEN TEMPERATURE AND FREQUENCY OF CONTRACTION IN THE TAIL-FURCAE OF BUCEPHALUS ELEGANS[1]

(Four figures)

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THE relation between temperature and various vital processes has been investigated extensively both in plants and animals. It was early known that biological processes are, within certain limits, accelerated by raising the temperature and retarded by lowering it. It was not until recently, however, that any attempt was made to study the phenomena quantitatively with the view of ascertaining the nature and the significance of the reactions involved.

The relation between temperature and the rate of chemical reactions, in terms of the van't Hoff rule is often expressed as follows:

$$\frac{k_0^{\left(\frac{10}{t_0-t_1}\right)}}{k_1}=Q_{10}$$

in which K_1 and K_0 are the velocity constants at temperatures t_1 and t_0, respectively, and Q_{10} the temperature coefficient for 10° intervals. Simply stated, this rule means that for every change of 10° the velocity of a reaction increases or decreases a certain number of times. For most chemical reactions the value of Q_{10} lies between 2 and 3.

Kanitz (1905) showed that increase in temperature results in increase in carbon dioxide assimilation in plants and that over a wide range of temperatures the value of Q_{10} lies between 2 and 3. Blackman (1905), in reference to the same phenomenon in plants, and

[1] Contribution from the Zoölogical Laboratory of The Johns Hopkins University, and the Mount Desert Island Biological Laboratory. This investigation was carried out under the direction of Professor S. O. Mast, whom the writer wishes to thank for his invaluable assistance.

Snyder (1905), in reference to the frequency of contraction in isolated terrapin heart, came to the same conclusion.

In an extensive review of the literature Kanitz (1915) maintains that his previous contention holds for a wide variety of phenomena, the most striking of which follow: speed of nervous impulse, frequency of pulsation of the contractile vacuole, rhythmical contraction of smooth muscle, respiration in mammals, response to light and gravity in plants, protoplasmic streaming, permeability, and developmental processes.

Several other investigators have since studied the effect of temperature on vital processes. Crozier (1924*a*, *b*) and 1926*a*), Crozier and Federighi (1925*a* and *b*), and Crozier and Stier (1925) in an extensive study of the effect of temperature on various vital processes have attempted by means of the Arrhenius equation to identify the reactions associated with these processes at different temperatures. The relation between these reactions and temperature is given by the Arrhenius equation as follows:

$$ln \frac{k_2}{k_1} = \frac{\mu}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

in which K_1 and K_2 equal the velocity constant at the two absolute temperatures T_1 and T_2, R is the gas constant for a given chemical reaction. They calculated the value of μ in this equation and they assume that in so doing they ascertained the energy necessary to change a molecule from the inactive to the active state. They maintain that their results show several "critical temperatures" each characterized by an abrupt change in the value of μ, and that the values of μ found in vital processes are similar to those found in specific catenary reactions, and they consequently conclude that in organisms there are, associated with responses to temperature, catenary reactions of the same kind.

Heilbrunn (1925) maintains that protoplasm does not behave as a homogeneous system and that the fact that Crozier does not take into account the effect of temperature on viscosity, coagulation permeability, and the like invalidates the interpretation of the meaning of changes in the value of μ in the Arrhenius equation.

Belehradek (1930) contends that critical temperatures probably do not occur in organisms, that many vital processes do not follow any given rule precisely, and that the Arrhenius μ is of no greater significance than the van't Hoff coefficient.

Krogh (1914), in a series of studies on the relation between temperature and the velocity of embryonic development of such forms as amphibia, fishes, insects, and echinoderms, maintains that in none of these forms can the relation be expressed even approximately by the van't Hoff formula but in all of these forms the relation is algebraic.

There is consequently considerable difference of opinion concerning the interpretation of the results obtained in observations on the effect of temperature on vital processes. It seems therefore highly desirable to extend these observations. For this reason a study of the effect of temperature on cercariae was made. Moreover, such a study is also of interest owing to the fact that nothing is known concerning the response to temperature in these organisms.

MATERIAL AND METHODS

The method of obtaining cercariae and the apparatus used to regulate temperature have already been described (Bevelander, 1933). Instead of using an observation aquarium, several pieces of glass tubing sealed at one end and bent so as to resemble a boot were used to confine the cercariae to a limited region while observations were made.

Observations on the frequency of contraction of the tail-furcae were made as follows: An actively swimming specimen was put into a glass boot together with about 1 cc. of water. Then one end of a short piece of a small rubber tube was slipped over the open end and a thermometer was inserted through the rubber tube into the glass boot. In this manner the bulb of the thermometer was firmly held in place only a few millimeters from the specimen under observation. The boot was now put into a water bath at room temperature (18°) and left at least fifteen minutes, after which the frequency of contraction in the tail-furcae was observed for one minute and recorded. The water in the water bath was then replaced with water from a reservoir approximately 3° lower than room temperature. This tem-

perature was maintained for fifteen minutes after which the frequency of contraction was again ascertained in the same way. This process was then successively repeated, the temperature being lowered approximately 3° each time, until the tail-furcae no longer

TABLE I

RELATION BETWEEN TEMPERATURE AND FREQUENCY OF CONTRACTION IN THE TAIL-FURCAE CF CERCARIAE

Interval between observations at different temperatures fifteen minutes. Observations were made in the order shown in the tables. Column headed $\frac{1}{T}$, reciprocal of absolute temperature; Log. of Av., logarithm of the average frequency; no, reading not taken.

Temperature in Degrees C.	Designation of Specimen Used					Average	$\frac{1}{T}$	Log. of Av.
	1	2	3	4	5			
	Contractions per Minute							
	Individual Results							
18	52	48	no	48	48	49.0	0.00346	1.69
14	36	32	34	36	32	34.0	0.00348	1.53
10	20	20	22	22	20	20.8	.00353	1.31
6	12	10	12	12	12	11.6	0.00354	1.06
3	5	2	0	3	5	3.0	0.00362	0.47
0	2	0	0	0	2	0.8	0.00367	0.03
— 2	0	0	0	0	0	0.0	.....	.....
0	0	0	0	0	0	0.0	.....	.....
5	7	6	0	7	6	5.2	0.00359	0.71
9	16	16	14	20	20	17.2	0.00354	1.23
14	33	32	36	36	34	34.2	0.00348	1.53
19	50	no	50	48	48	49.0	0.00338	1.70
22	64	64	68	66	no	65.5	0.00339	1.81
25	98	94	88	96	no	94.0	0.00335	1.97
28	100	98	104	106	no	102.0	0.00333	2.00
32	80	78	80	76	no	78.5	0.00326	1.89
38	12	14	15	20	no	15.2	.....	.....
40	0	0	0	0	no	0.0	.....	.....

showed any signs of movement. Then the temperature was raised approximately 3° and the process successively repeated until the temperature became so high that the tail-furcae no longer contracted. In this way the frequency of contraction was ascertained in each of 18 specimens in temperatures ranging from 0° to 40°. The results obtained are presented in Tables I and II and Figures 1 and 2.

FREQUENCY OF CONTRACTION IN THE TAIL-FURCAE

Examination of Table I, a protocol for five experiments, shows that, as the temperature decreased from 18° to 0°, the average frequency of contraction of the tail-furcae decreased from 49 to 0.8 per minute, and that, as the temperature increased from 0° to 40°, the frequency increased from 0 to a maximum of 102 at 28°, after which it rapidly decreased to 0 at 40°. This change in frequency is typical for all the experiments performed. It is clear, therefore, that temperature has a very decided effect on the frequency of contraction in the tail-furcae of cercariae.

Figure 1 shows more clearly the relation between temperature and the frequency of contraction in the tail-furcae. The curve in this figure is sigmoid in nature, similar to those so frequently obtained for biological reactions.

Table II which contains a summary of the results obtained with 18 specimens shows that as the temperature increased from 3° to 40° the van't Hoff temperature coefficient decreased from 29.8 to 0.0. This indicates that the processes associated with contraction vary continuously with the temperature.

It has been maintained that, when the logarithm of the rate or the frequency observed in biological phenomena is plotted against the reciprocal of the absolute temperature and a straight line results, the value of the Arrhenius μ is constant and the processes involved in the phenomena are constant, and that, when more than one straight line occurs, the value of μ is not constant, and there are critical temperatures at the points at which the lines cross.

The logarithm of the average frequency of contraction of 18 cercariae in different temperatures was plotted against the reciprocal of the absolute temperature and the results obtained are presented in Figure 2. This figure shows that the curve thus produced is not a straight line or a series of straight lines. It consequently indicates that the value of μ and the processes involved vary with the temperature.

ADAPTATION AND RECOVERY

An actively swimming cercaria was put into a glass boot, then a thermometer was inserted, after which the boot was put into the

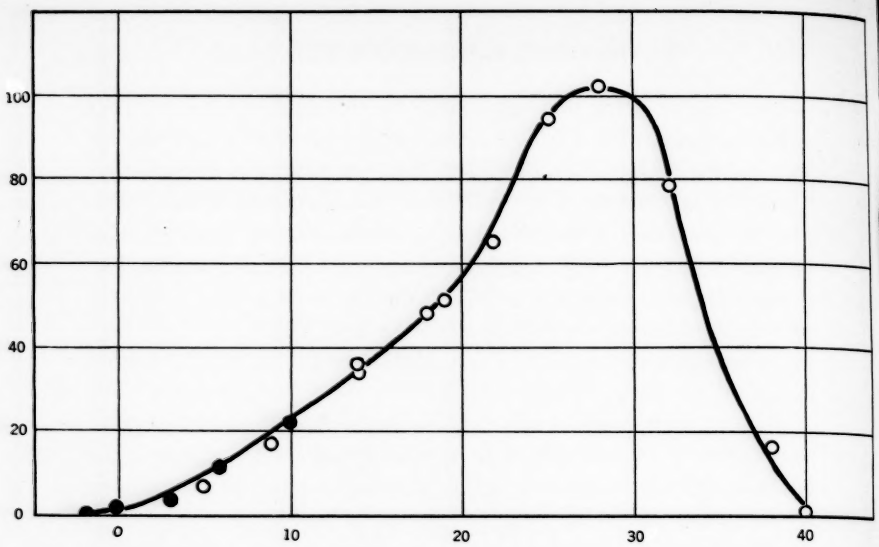

FIG. 1.—Relation between temperature and frequency of contraction. Ordinates, frequency; abscissae, temperature; dots, frequency after lowering the temperature; circles, frequency after raising it. Each point on the curve is an average of five specimens.

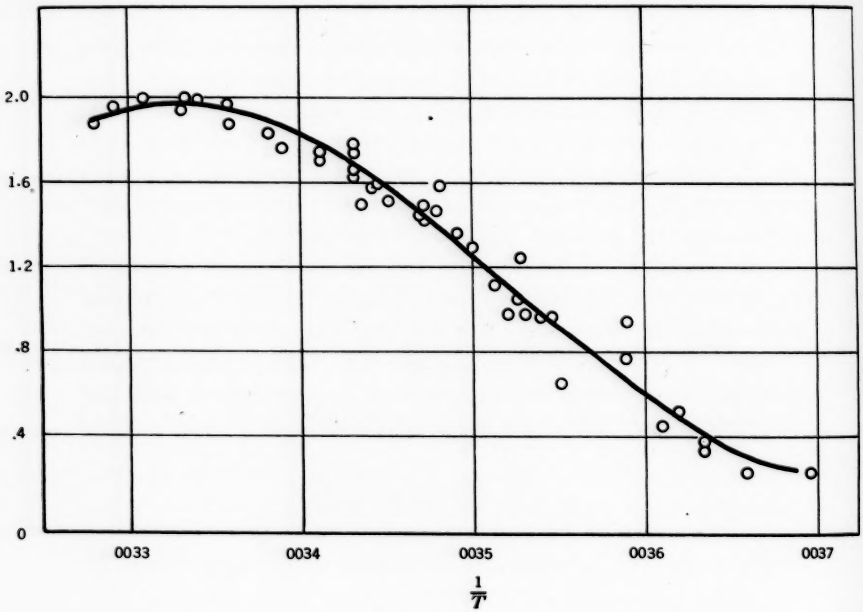

FIG. 2.—Curve showing the relation between temperature and frequency of contraction. Ordinates, logarithm of frequency; abscissae, reciprocal of absolute temperature. Each point on the curve represents an average of five readings made on eighteen specimens.

water bath at room temperature, left fifteen minutes, and the number of contractions in the tail-furcae in one minute ascertained. Immediately following this, the temperature was decreased to 4°, held for one minute, and then raised to room temperature. The frequency of contraction was now lower than it had been before the temperature was lowered. The time required to regain the former frequency was now ascertained; then the temperature was again decreased to 4°, held two minutes, and then raised to room temperature, after

TABLE II

AVERAGE RESULTS OF EIGHTEEN SPECIMENS SHOWING RELATION BETWEEN TEMPERATURE, AVERAGE FREQUENCY, AND TEMPERATURE COEFFICIENT

Temperature (Degrees)	Average Frequency	Q_{10}
3	0	.....
5	2.2	.....
10	12.0	29.75
15	31.8	7.02
17	38.0	2.43
19	50.1	3.98
23	78.0	3.02
26	87.8	1.48
28	100.0	1.92
31	89.6	0.69
36	43.6	0.24
38	13.5	0.00

which the time required to regain the original frequency was again ascertained. This was successively repeated leaving the cercaria longer and longer in the low temperature until an interval of ten minutes was reached. Then the whole process was repeated with 4 other specimens. It was now repeated with each of 6 different specimens subjected to different temperatures for the same length of time in place of the same temperature for different lengths of time. The results obtained are presented in Tables III and IV and Figures 3 and 4.

Table III shows that the longer the cercariae were left in the low temperatures, the longer they required to recover, the time for recovery being 1 min. 43 sec. if left one minute at 4°, and 9 min. 49

TABLE III

RELATION BETWEEN EXPOSURE TO 4° FOR VARYING INTERVALS AND THE TIME REQUIRED FOR RECOVERY

Exposure Time in Minutes at 4°	Designation of Specimens					Average
	1	2	3	4	5	
	Recovery Time in Minutes					
	Individual Results					
1	1′30″	1′40″	1′48″	1′56″	1′42″	1′43″
2	2′10″	2′35″	2′21″	2′18″	2′5″	2′18″
3	2′48″	2′55″	2′50″	2′30″	2′58″	2′48″
4	3′52″	4′15″	4′18″	4′25″	3′45″	4′7″
5	4′42″	4′30″	4′50″	5′5″	5′	4′49″
6	6′	6′12″	6′10″	6′20″	6′24″	6′13″
8	7′44″	8′	7′25″	7′35″	7′52″	7′43″
10	9′30″	10′24″	9′25″	9′5″	9′52″	9′39″

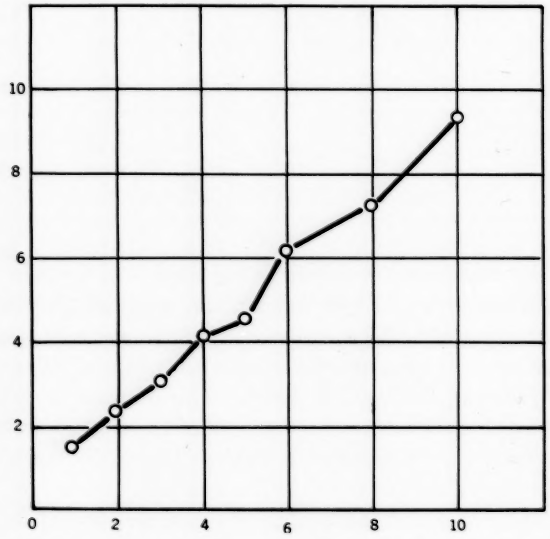

FIG. 3.—Relation between exposure of cercariae at 4° for different intervals, and the time required to recover at room temperature (17°).

sec. if left 10 minutes. This indicates that recovery time is nearly equal to the exposure period. This is more clearly shown in Figure 3.

Table IV shows that as the temperature at which the specimens were left ten minutes decreased or increased from room temperature, the time required to recover at room temperature increased. It also shows that the time required to recover after exposure at 5° is nearly

TABLE IV

RELATION BETWEEN EXPOSURE FOR TEN MINUTES AT VARIOUS TEMPERATURES, AND THE TIME REQUIRED TO RECOVER AT ROOM TEMPERATURE

Exposure Temperature (Degrees)	Designation of Specimens						Average
	1	2	3	4	5	6	
	Recovery Time in Minutes						
	Individual Results						
5	6′50″	6′07″	6′22″	6′46″	6′00″	6′25″	6′25″
9	3′30″	3′20″	3′25″	3′40″	3′15″	3′25″	3′25″
12	1′55″	2′15″	2′05″	2′12″	2′02″	2′05″	2′05″
17							
20	1′40″	1′20″	1′27″	1′36″	1′41″	1′30″	1′32″
25	2′05″	2′15″	2′00″	2′08″	2′18″	2′10″	2′09″
30	4′00″	4′09″	4′17″	4′05″	4′10″	4′09″	4′08″
35	4′45″	4′35″	4′18″	4′33″	4′40″	4′35″	4′34″

six times as great as it is after exposure at 12°, and that after exposure to 35° it is nearly four times as great as it is after exposure to 20°. Figure 4 shows this relation even more clearly. It shows that from 5° to 17° the time for recovery is inversely proportional, and that from 17° to 35° it is directly proportional to increase in temperature.

One noticeable difference between adaptation at high and low temperatures in cercariae is the effect on reversibility in recovery. Specimens usually recover from exposure to low temperature (0°, e.g.) without any noticeable effect, that is, reversibility is complete. At high temperatures, however, this does not obtain.

DISCUSSION

As previously stated, the effect of temperature on biological processes has been studied by many investigators who seem to differ in their interpretation of the phenomena. Some investigators maintain that their data are accurately expressed by the van't Hoff equation; others employ the Arrhenius formula; special equations either algebraic or exponential are also used to some extent. Another group

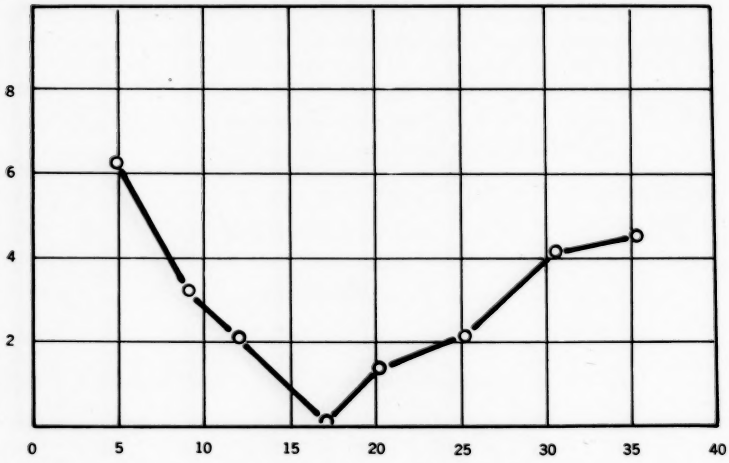

FIG. 4.—Relation between temperature to which cercariae had been subjected for ten minutes, and recovery time at room temperature (17°).

holds that in dealing with a heterogeneous system such as protoplasm that temperature affects several processes with different velocities and accordingly the meaning of temperature coefficients is of little value. If this latter idea is correct, then the data do not represent the effect of temperature upon any one process, but on several processes which occur simultaneously.

Temperature changes in cercariae undoubtedly affect several physico-chemical changes in the organism at different velocities. It seems quite probable that the longitudinal and circular muscles in the furcae would react with different velocities and that changes in viscosity, coagulation, permeability, and the like would also be

affected differently at different temperatures. Thus, it does not seem at all unreasonable to find that the data presented for cercariae do not lend themselves very well to formulation over a wide range of temperatures. It is of interest to note, however, that, when the data are represented graphically, a sigmoid curve results, as is so frequently the case in experiments of this kind.

SUMMARY

1. Temperature affects the frequency of contraction of the tail-furcae very markedly. As the temperature increases from 0°, the frequency of contraction increases from 0 to a maximum of 102 at 28°, after which it rapidly decreases to 0 at 40°.

2. The temperature coefficient decreases from 29.8 at 3° to zero at 38°. This indicates that the processes associated with change in temperature vary throughout the whole range investigated. The evidence at hand does not indicate the occurrence of "critical temperatures."

3. If the temperature is decreased and held for a given length of time and then increased again, the frequency of contraction in the tail-furcae is much lower than it was and the time required for recovery depends specifically upon the extent of the decrease in temperature and the time the cercariae are exposed to it.

4. If specimens are exposed to temperatures between 1° and 4° the frequency is completely reversible. If they are exposed at 40° the frequency is incompletely reversible or not reversible at all, depending upon the time of exposure.

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THE EARLY DEVELOPMENT OF SPONTANEOUS AND REFLEX BEHAVIOR IN CAT EMBRYOS AND FETUSES

(Three figures)

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INTRODUCTION

THE spontaneous movements of embryos and fetuses, observed upon opening the uteri of previously decerebrated cats, were reported recently (Windle and Griffin, 1931). The smallest specimens in which any motility was detected were 16 mm. long. The motility was a simple ventrolateral flexion of the head, believed to have been induced by a pressure stimulus at the time of removal. Progressive cephalocaudal development of spontaneous movements was studied in a series of specimens and it was thought that, in general, the complex activities and unit reflexes of older fetuses individuate from the simple, total mass movements of the embryo.

During the last two years, observations of embryonic and fetal behavior have been continued as opportunities have arisen and the earlier studies can now be supplemented by another series. In the previous experiments, every precaution was taken to avoid disturbing the embryos by unnecessary handling. Stimulation with brush and probe was resorted to only after time had been allowed for studying the "spontaneous" activity that might present itself. In most of the present experiments, every effort was directed toward prompt study of "reflex" behavior. This fact should be borne in mind throughout because, in all probability, it has a direct bearing on some striking differences between the two series of observations.

MATERIAL AND METHODS

Pregnant cats were subjected to decerebration (under ether anesthesia) in order that they might be opened later and the fetuses

[1] Contribution No. 182.

studied without resorting to the use of drugs which would interfere with fetal movements. In the previous experiments, transection of the midbrain was the method of choice. In all but a few of the present experiments decerebrate rigidity was produced by the anemia method of Pollock and Davis (1924). Since it was usually accomplished with the loss of scarcely a cubic centimeter of blood, the condition of the fetuses was thought to have been better than could have been expected by the former method. The operated animals were allowed to lie quietly for an hour or more—usually two (Table I). During this time the body temperature was maintained (when necessary, by warmed towels and a lamp) at about 36.5°–38.5° C. At the end of the period allowed for recovery from the ether and when all was in readiness for observations, the abdomen was quickly opened, one vesicle of the uterus was incised, and the embryo in its amnion and with circulation as nearly as possible intact examined either with the naked eye or with the aid of a 3× dissecting binocular strapped to the head of the observer.

Stimulation of the embryo was accomplished in a number of ways. The usual procedure was to tap the amnion over one part or another by means of a blunt metal, bone, or glass instrument. After this, in the small specimens, direct stimulation of the embryo was attempted by inserting a very thin steel needle or a slender cactus spine into the amniotic cavity where the specimen could be touched, pricked, or pressed upon. The order of procedure was varied in different cases and more extensive experimentation was possible in the larger specimens. While one embryo was being examined the other segments of the uterus were kept as warm as possible within the abdominal cavity. In nearly all experiments, two and, in some of them, three observers and a stenographer were employed. The duration of each examination was very short: thirty seconds to a minute or more for the 12 mm.—16 mm. specimens, several minutes for those 18 mm.—28 mm., and five to fifteen minutes for the larger ones. In the case of the larger specimens it was possible to transfer fetus and placenta to the warmed saline solution for study, after a preliminary examination *in situ*.

Observations of induced or spontaneous movements were recorded for most of the 178 embryos and fetuses of 44 decerebrated cats.

TABLE I

LIST OF MATERIAL ARRANGED ACCORDING TO THE AVERAGE SIZE OF EACH LITTER

Index No.	Litter No.	Number of Embryos	Days since Mating	Size in mm.	Time Allowed for Recovery from Ether
1	5:32	2	18	5.5–6	Not decerebrated
2	77:31	4	19	7	Not decerebrated
3	78:31	4	20	8–9	Not decerebrated
4	70:31	4	21	10	Not decerebrated
5	83:31	4	?	11	Not decerebrated
6	3:32	5	?	11–12	2 hrs. 15 min.
7	8:32	4	?	11–12	2 hrs.
8	76:31	3	22	11.5–12	1 hr. 10 min.
9	40:32	4	?	12	2 hrs.
10	15:32	5	?	12–13	1 hr. 55 min.
11	37:32	3	23	13	2 hrs. 10 min.
12	12:32	4	?	13–13.5	2 hrs.
13	6:32	4	?	13–14	2 hrs..
14	9:32	4	?	13–14	2 hrs.
15	29:32	4	?	14	1 hr. 30 min.
16	25:32	2	?	14	2 hrs. 40 min.
17	13:32	5	?	13–14.75	1 hr. 40 min.
18	32:32	4	23.5	13.5–14.5	2 hrs. 45 min.
19	33:32	4	23.5	13.5–14.5	2 hrs.
20	10:32	5	?	14–14.5	2 hrs.
21	36:32	5	?	14–15	2 hrs. 15 min.
22	25:31	4	?	14–15	2 hrs. 35 min.
23	38:32	4	24	14–15.5	2 hrs.
24	84:31	4	?	14.5–15	2 hrs. 40 min.
25	30:32	2	?	15–16	2 hrs. 30 min.
26	34:32	4	?	15.5–16.5	2 hrs.
27	26:32	6	?	16–17	2 hrs.
28	35:32	3	?	16+–18	2 hrs.
29	39:32	5	?	18	2 hrs.
30	18:32	4	?	18.5	2 hrs.
31	27:32	4	?	18.5	2 hrs.
32	85:31	4	?	21	2 hrs. 45 min.
33	20:32	3	?	21–23	2 hrs.
34	14:32	3	?	25	1 hr. 45 min.
35	90:31	4	29	26–28	1 hr. 10 min.
36	19:32	5	?	28–30	1 hr.
37	95:31	4	30	30	3 hrs. 30 min.
38	17:32	6	?	30	2 hrs. 45 min.
39	28:32	4	?	30	2 hrs.
40	86:31	5	?	34	2 hrs. 25 min.
41	1:32	4	?	35	2 hrs. 30 min.
42	22:32	7	?	36	2 hrs. 30 min.
43	16:32	3	?	38	3 hrs. 15 min.
44	21:32	2	?	38	2 hrs. 45 min.
45	23:32	4	?	38	2 hrs.
46	11:32	4	?	40	2 hrs.
47	4:32	3	?	40	2 hrs.
48	7:32	5	?	42	2 hrs.
49	24:32	5	?	45	2 hrs.

The specimens used in the experiments are listed in Table I which includes a few premotile embryos not to be considered at present (first 5 cats).

OBSERVATIONS

The following experiments have been summarized whenever possible and grouped according to age of the specimens and character of their movements. In the cases in which mating was not observed, ages have been computed from an age-length curve (Windle and Griffin, 1931).

TWENTY-TWO TO TWENTY-FOUR DAYS

Two litters (index 6 and 7; Table I) of embryos of unknown age and one (index 8) obtained at the end of the twenty-second day after an observed mating were examined. All these specimens were 11 mm.–12 mm. greatest length (Fig. 1). No spontaneous somatic movements could be observed. All that was seen was a rhythmic passive movement of the head in response to the beating of the heart. No reflexes could be elicited from the specimens of the first two litters; those of the third were not stimulated intentionally. Tapping the amnion with a metal instrument caused passive displacement of the embryo and nothing more. Touch and pressure brought to bear upon the embryos by inserting a steel needle through the amnion caused no movements at all. A fourth litter (index 10; Table I), comprising one 12 mm. and four 13 mm. specimens and a fifth (index 19), 13.5 mm.–14.5 mm. long, likewise showed no spontaneous movements and no reflexes. In addition to these premotile specimens, the first, third, and fourth 12 mm. embryos of litter 40:32 (index 9; Table I) exhibited neither spontaneous nor reflex movements. On the other hand, the second specimen (line 2; Table II) gave positive results from stimulation but showed no spontaneous activity. Immediately after this embryo had been brought into view the amnion was tapped lightly several times in succession over the foreleg and neck region with a slender glass rod. A very slight active abduction or flexion of the foreleg, quite distinct from any passive oscillation, was evident. This was seen through the dissecting binocular by one observer and, independently, by the unaided eye of a second one. Further stimulation gave negative results.

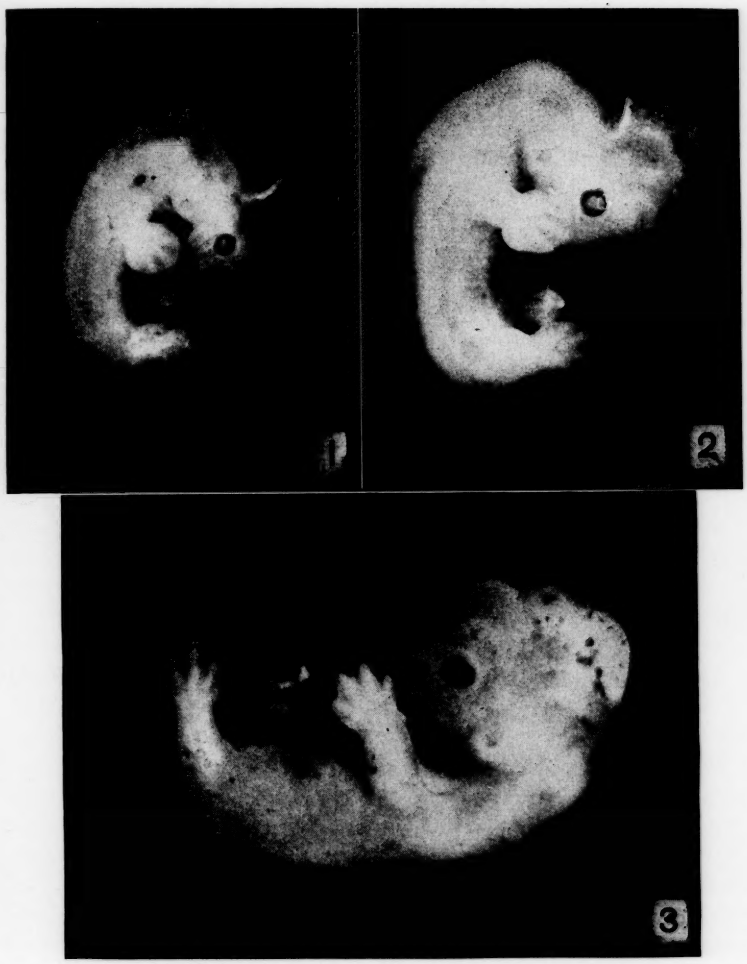

FIG. 1.—An 11-millimeter cat embryo of litter 3:32. This is probably near the end of the twenty-second day after insemination, shortly before the earliest movements can be elicited but at least a full day before spontaneous motility appears. Photographed in saline solution before fixation. ×4.

FIG. 2.—A 14-millimeter cat embryo of litter 6:32, at least 23 days after insemination. This embryo showed no spontaneous movements but abduction of the forelimb and possibly a slight movement of the neck was elicited by stimulation. Photographed in saline solution before fixation. ×4.

FIG. 3.—An 18½-millimeter embryo of litter 18:32, about 25 days after insemination. This embryo showed dorsolateral flexion and rotation of the head and neck co-ordinated with foreleg flexion spontaneously. Local unit movements could be elicited in the forelegs and hind legs as well as in the lower part of the trunk. Photographed in saline solution before fixation. ×4.

TABLE II

MOVEMENTS OBSERVED IN 12 MM.–14.75 MM. CAT EMBRYOS. THE LIST HAS BEEN ARRANGED ACCORDING TO SIZE OF SPECIMENS IRRESPECTIVE OF LITTER*

Line No.	Index No. in Table I	Litter No.	Embryo No.	Embryo Size (mm.)	Spontaneous Movements		Induced Movements	
					Foreleg	Head-Neck	Foreleg	Head-Neck
1.....	9	40:32	1	12	–	–	–	–
2.....	9	40:32	2	12	–	–	+	–
3.....	9	40:32	3	12	–	–	–	–
4.....	9	40:32	4	12	–	–	–	–
5.....	10	15:32	1	12	–	–	–	–
6.....	10	15:32	2	13	–	–	–	–
7.....	10	15:32	3	13	–	–	–	–
8.....	10	15:32	4	13	–	–	–	–
9.....	10	15:32	5	13	–	–	–	–
10.....	11	37:32	1	13	–	–	+?	–
11.....	11	37:32	2	13	–	–	+	–
12.....	11	37:32	3	13	–	–	–	–
13.....	12	12:32	1	13	–	–	–	–
14.....	12	12:32	2	13	–	–	+	?
15.....	13	6:32	2	13	–	–	–	–
16.....	13	6:32	3	13	–	–	–	–
17.....	13	6:32	4	13	–	–	–	–
18.....	14	9:32	1	13	–	–	+	?
19.....	14	9:32	3	13	–	–	–	–
20.....	17	13:32	2	13	–	–	+?	–
21.....	17	13:32	3	13	–	–	+	–
22.....	12	12:32	3	13.5	–	–	+	?
23.....	12	12:32	4	13.5	–	–	–	–
24.....	18	32:32	1	13.5	–	–	+	–
25.....	18	32:32	3	13.5	–	–	+	–
26.....	19	33:32	1	13.5	–	–	–	–
27.....	19	33:32	4	13.5	–	–	–	–
28.....	21	36:32	3	13.5	–	–	–	–
29.....	13	6:32	1	14	–	–	+	?
30.....	14	9:32	2	14	–	–	+	?
31.....	14	9:32	4	14	–	–	–	–
32.....	15	29:32	1	14	–	–	+	–
33.....	15	29:32	2	14	–	–	+	–
34.....	15	29:32	3	14	–	–	+	–
35.....	15	29:32	4	14	–	–	+	–
36.....	16	25:32	1	14	–	–	+	?
37.....	16	25:32	2	14	–	–	+	–
38.....	17	13:32	4	14	–	–	+	+?
39.....	17	13:32	5	14	–	–	+	+?
40.....	18	32:32	2	14	–	–	+	–
41.....	19	33:32	3	14	–	–	–	–
42.....	21	36:32	1	14	–	?	+	–
43.....	21	36:32	4	14	–	–	?	–
44.....	22	25:31	1	14	–	–	o	o
45.....	22	25:31	2	14	–	–	o	o
46.....	23	38:32	1	14	–	–	+	+
47.....	23	38:32	4	14	–	–	–	+
48.....	24	84:31	1	14	–	–	o	o
49.....	18	32:32	4	14.5	–	–	+	?
50.....	19	33:32	2	14.5	–	–	–	–
51.....	20	10:32	2	14.5	–	–	+	+
52.....	20	10:32	3	14.5	–	–	+	+
53.....	20	10:32	4	14.5	–	–	+	+
54.....	20	10:32	5	14.5	–	–	+	+
55.....	24	84:31	2	14.5	+	–	o	o
56.....	17	13:32	1	14.75	+?	–	+	+?

* The plus and minus signs indicate presence and absence of movements. The zero is used when no observations were made. Doubtful responses are indicated by the question mark.

The 13 mm.–14.75 mm. embryos in Table II are probably all 23 days or 24 days old. The reactions of most of them are similar enough to be grouped. In only one specimen (line 55; Table II) were definite spontaneous movements observed. This specimen measured 14.5 mm. greatest length and two of its litter mates were 15 mm. long. The movements consisted of very small flexions of the foreleg as a whole; no active head movement could be detected. A slight twitch of one foreleg of a 14.75 mm. specimen (line 56; Table II) was seen; this may have been caused by handling and its spontaneity was regarded as questionable. One observer was of the opinion that a slight extension of the head occurred spontaneously in one 14 mm. embryo (line 42; Table II) but it was very questionable. In no other embryo of this group was any spontaneous activity found. Of course, in most of the cases, no time was lost in waiting for possible latent behavior to manifest itself. But, in earlier experiments (Windle and Griffin, 1931) and a few critical ones in which this was done in the present series, no spontaneous movements were seen in any specimen smaller than 14.5 mm.

There is no doubt that responses to stimulation can be detected before spontaneous movements appear. Some of the members of nearly all litters listed in Table II showed movements of the foreleg. These could be elicited by tapping the foreleg, shoulders, or neck lightly before the amnion was torn away. The type of instrument used for this purpose was of no consequence; usually the tip of the scalpel was employed, but often a bone or glass rod was used. They could be elicited by passing a needle (metal or fiber) through the amnion and touching or pressing lightly on the limb several times in succession with the flat side of the instrument. A single stimulus was usually ineffectual in the smallest specimens but often sufficed to produce movements in the larger ones. The movement which resulted was distinctly an active one; it was never confused by any observer with the passive oscillation resulting from displacement and release of the limb. Since it was more rapid than the passive movements, it might be described as a slow twitch of the leg. The contraction was usually confined to the muscles of the limb itself and did not involve the neck muscles. In a few 13 mm. and 14 mm. embryos (Fig. 2), the shoulder muscles could be made to contract and the resultant movement sometimes appeared to involve the neck.

However, neck movement of this kind was so very slight that it was recorded as doubtful (?, in Table II).

No positive head-neck flexion, independent of shoulder movements, could be elicited by stimulation of the head in any of the smaller embryos in this group (Table II). Very slight independent head extension was thought to have been seen momentarily in two 14 mm. and one 14.75 mm. specimens of litter 13:32 (indicated by +? in Table II). Only six specimens (lines 46, 47, 51, 52, 53, and 54; Table II) 14 mm.—14.5 mm. long exhibited distinct extension of the head backward and to one side. All but one that gave any indication of head movements likewise exhibited isolated, independent contractions in the foreleg; these were more distinct and executed less sluggishly in the 14.5 mm. specimens than in the smaller ones. No amount of stimulation of the head produced distinct head movements in embryos smaller than 14 mm. long. Neither could movements of the hind leg and lower trunk be elicited, even though the hind-limb buds in the larger ones were as large as the forelimb buds in the smaller embryos.

That we are not confusing a passive phenomenon with true active movement in case of the primitive abduction of the foreleg, is strikingly illustrated by comparing litters 32:32 and 33:32. The two cats (index 18 and 19; Table I) from which these litters were obtained were very different in appearance. The first was the common "alley" type; the second had shorter, broader head, tail, and legs and, in general, was of stocky build with longer, thicker fur than the first one. Both animals were mated on the same evening by the same male and were operated within a half hour of each other. The embryos were identical; each uterus contained two 13.5 mm., one 14 mm., and one 14.5 mm. specimen. None of those of the second cat (33:32) showed any movements; tapping the amnion resulted in very slight rebound of the embryo in each case, but it was impossible to confuse this with active motility. All four specimens from the first cat (32:32) exhibited typical contractions of the foreleg musculature and it was thought that slight neck movements could be observed in the largest one when the head was tapped with a slender glass rod. These experiments indicate that size is not the only factor determining the time at which motility is established in the embryo.

TWENTY-FOUR TO TWENTY-FIVE DAYS

The movements of embryos 15 mm.–15.5 mm. long (lines 57–66; Table III) were very similar to those of the 14.5 mm.–14.75 mm. specimens. Four exhibited spontaneous foreleg movements at the

TABLE III

MOVEMENTS OBSERVED IN 15 MM.–18.5 MM. CAT EMBRYOS. THE LIST IS ARRANGED ACCORDING TO SIZE OF SPECIMENS IRRESPECTIVE OF LITTER. THEY ARE BETWEEN 24 AND 25 DAYS OLD*

Line No.	Index No. in Table I	Litter No.	Embryo No.	Embryo Size in mm.	Spontaneous Movements				Induced Movements			
					Foreleg	Head-Neck	Lower Trunk and Tail	Hind Leg	Foreleg	Head-Neck	Lower Trunk and Tail	Hind Leg
57	21	36:32	2	15	+	+	−	−	+	+	−	−
58	22	25:31	3	15	−	−	−	−	+	+?	−	−
59	22	25:31	4	15	−	−	−		−	−	−	−
60	23	38:32	2	15	−	?	−	−	+	+	?	−
61	24	84:31	3	15	+	?	−	−	o	o	o	o
62	24	84:31	4	15	+	−	−	−	o	o	−	−
63	25	30:32	2	15	−	−	−	−	−	−	−	−
64	23	38:32	5	15.5	+	+	−	−	+	+	−	−
65	26	34:32	2	15.5	−	−	−	−	+	−	−	o
66	26	34:32	3	15.5	−	−	−	−	+	−	−	−
67	25	30:32	1	16	−	+	−	−	+	+	−	−
68	27	26:32	1	16	−	−	−	−	+	+	+?	−
69	27	26:32	4	16	−	−	−	−	+	+	o	o
70	28	35:32	1	16+	+	+	−	−	+	+	−	−
71	26	34:32	4	16.5	−	+	−	−	+	−	−	−
72	27	26:32	3	16.5	−	−	−	−	+	+	−	−
73	27	26:32	6	16.5	−	−	−	−	+	+	+	?
74	27	26:32	2	17	+	+	−	−	+	+	o	o
75	27	26:32	5	17	−	−	−	−	+	+	o	−
76	28	35:32	2	17	+	+	−	−	+	+	−	−
77	29	39:32	4	17	−	−	−	−	+	−	−	−
78	29	39:32	5	17.5	−	−	−	−	+	−	+	−
79	28	35:32	3	18	+	+	−	−	+	−	−	−
80	29	39:32	1	18	+	+	−	−	+	−	−	+
81	29	39:32	2	18	+	+	+	−	+	+	+	+
82	29	39:32	3	18	+	+	−	−	+	+	+	+
83	30	18:32	1	18.5	+	+	−	−	+	+	+	+
84	30	18:32	2	18.5	+	+	−	−	+	+	+	+
85	30	18:32	3	18.5	+	+	−	−	+	+	+	+
86	30	18:32	4	18.5	+	+	−	−	+	+	+	+
87	31	27:32	1	18.5	+	+	−	−	+	+	+	−
88	31	27:32	2	18.5	+	+	−	−	+	+	+	?
89	31	27:32	3	18.5	+	+	−	−	+	+	o	o
90	31	27:32	4	18.5	+	+	−	−	+	+	+	+?

* See footnote on page 526.

time they were removed. These usually appeared as slight forward-backward flexions, unassociated with any other muscular contractions. However, in two specimens, they were sometimes associated with slight turning or backward extension of the head. Very fleeting spontaneous motility of the neck was thought to have been observed

in two other embryos (? in lines 60 and 61; Table III). The evidence of spontaneous muscular contractions in embryos smaller than 16 mm. long definitely suggests that movements of the foreleg (5 unquestionable cases) appear before contractions become distinct in the neck muscles (two unquestionable cases). This is in agreement with the observations on movement occurring in response to stimulation in the smaller specimens where the ratio is 33 to 8 in favor of the leg muscles. All but two of the 15 mm.–15.5 mm. embryos which were stimulated showed very distinct local movements of the foreleg. More often than not, they were independent of any other muscular contraction but sometimes they were followed by head movements. In one case a slight trunk flexion may have been elicited by tapping the amnion over the lower trunk. Very rarely did foreleg movement follow an initial head extension and when it did the leg movement seemed to be produced passively by the neck muscles.

Few spontaneous movements were seen in embryos 16 mm.–17.5 mm. long (lines 67–78; Table III). Three specimens showed foreleg motility, but this was associated with contraction of neck muscles as a mass action. Two others showed only head movements. More of the embryos might have been found to exhibit spontaneous behavior if the attention of the investigators had been directed toward this rather than toward observing contractions in response to stimulation.

Movements were much easier to elicit in 16 mm.–17.5 mm. embryos than in the smaller ones; often a single light touch with a needle sufficed to produce local muscular contractions or one tap of the amnion was enough to produce a more massive movement. All embryos stimulated exhibited foreleg movements and, in most of those in which tests were made, head movements could be elicited. Touching the foreleg (light pressure) usually resulted in a local movement in the neighborhood of the point of application of the stimulus. Sometimes this movement was the only one observed; at other times a dorsolateral extension of the head followed it and, as a consequence of this, the foreleg as a whole was carried forward slightly. When the head or neck was tapped lightly a head-neck movement often followed and this was frequently accompanied by a (passive or secondary) forward extension of the foreleg on the same side; it was never followed by local movements in the foreleg. These observations sug-

gest that impulses may be transmitted rostrad more readily than caudad in the cervical region of the nervous system. The embryos which showed no head movements (lines 71, 77, 78, and 79; Table III) were the last ones of their litters studied and were sluggish because the uterus had cooled to some extent. Thus the persistence of the older, local foreleg movements after failure of more recently acquired neck-muscle contractions is demonstrated. This was confirmed in the studies on other embryos which showed both leg and head movements at the beginning of experiments and only those of the leg at the end.

A local contraction of the lower trunk muscles was produced in three embryos by tapping the caudal end of the specimens. In two (lines 73 and 78; Table III), it was very definite; in the other (line 68; Table III), very fleeting. It was thought to have been observed in a third specimen 15 mm. long but was doubtful (line 60; Table III). When the lower trunk was flexed the hind legs were carried forward passively; no movements in the forward parts of the body followed. Stimulation of the forward parts never caused lower trunk movements.

Definite improvement of spontaneous and elicited movements was observed in 18 mm. and 18.5 mm. embryos. All specimens of this size (Fig. 3) exhibited spontaneous movements of the head and foreleg at the time they were brought into view. The head movement consisted of dorsal extension, abduction, and a combination of these (rotation). It was accompanied by forward and backward movements of the forelegs which seemed to be produced by the same mass contractions that moved the head. Independent local movements were not observed spontaneously in the foreleg. The mass movement of the neck and forelimb extended to the lower part of the trunk in one 18 mm. specimen and caused the hind legs to be carried forward passively (line 81; Table III).

The movements of 18 mm.–18.5 mm. embryos elicited by stimulation were very definitely of two types. In the first place, touching an embryo with a metal or fiber needle usually produced a local, isolated contraction of the muscle groups in the vicinity. This was sometimes followed by more general movements (see *infra*), but was often independent. Simple quick contractions of foreleg muscles

could always be elicited in this way; they resulted in local movements within the limb, such as flexion at the wrist with extension at the elbow, in a number of instances. Similarly, in a few, a quick jerk of the head dorsad followed touching the tip of the nose with the point of the needle; this movement was usually succeeded by the more general mass reaction. Local hindleg movements (abduction) like those in the forelimb could be obtained in a number of specimens; they were smaller than the ones seen in the forelegs of the same embryo—more like the foreleg movements in younger embryos. A quick isolated contraction of the lower trunk was obtained occasionally after touching the caudal end of the embryo.

A second type of motility was encountered. This was a slower, more massive, generalized reaction comparable with the spontaneous movements. It could be elicited with great ease by pressing on the embryos, tapping the amnion, or disturbing them grossly in any way. It began as a dorsolateral extension and rotation of the head and neck and extended to the forelimb which was carried forward as a whole. A small amount of forward flexion of the neck or upper part of the trunk was involved in this movement. In only one case did this mass reaction extend as far caudad as the lower limbs. It usually followed the local backward jerk of the head produced by stimulating the nose; it often followed local reflexes in the forelimb, and, once in a while, was observed after stimulation of the hind limb or lower trunk. It is important to emphasize the fact that this slow, massive wave of movement was confined to the rostral half of the body in all cases except one, in which it did pass as far caudad as the posterior limbs. No movements in the lower part of the body could be elicited by stimulation of regions farther rostrad. Nevertheless, stimulation of the caudal parts was followed by motility of more rostral ones. Here, again, we have evidence that impulses pass rostrad in the lower part of the nervous system before they do caudad. Stimulation of one fore or hind limb was sometimes followed by movement of the opposite limb before the descending wave of mass-movement appeared.

TWENTY-SIX TO THIRTY DAYS

Spontaneous somatic movements of 21 mm.–30 mm. cat fetuses were of greater magnitude and longer duration than those of smaller

ones. Dorsoventral and lateral movements of the head to one side and the other were observed in all specimens. The combination of these motions produced rotation of the head which became more pronounced in the larger specimens than it was in smaller ones. The spontaneous head movements sometimes occurred independently in 21 mm. embryos but usually they were followed by other activities and appeared to be part of a general mass-reaction. The neck and fore part of the trunk were usually involved with the head in all cases; the lower trunk, although flexed at times in most, was not rotated in any smaller than 30 mm. long. Flexion of the tail occurred somewhat earlier than rotation of the lower part of the trunk (Table IV). The head-neck-trunk movements began to assume a bilateral character in the 21 mm. fetuses and were distinctly "fish-like" in 28 mm.– 30 mm. specimens. Gross flexion and extension of the foreleg at the shoulder accompanied the mass-movement of the trunk in every case but distinct flexion at the hip did not appear spontaneously in any smaller than 26 mm. long. Flexion at the elbow and wrist was observed at times in many specimens larger than 25 mm. long, but movements at the knee occurred only after stimulation. Jaw and tongue motion appeared spontaneously as indicated in Table IV. The combination of foreleg movements with the repeated head-trunk mass undulations produced a composite activity which could be approximately described as "writhing" or "squirming." This behavior complex was not executed completely every time it was begun. Often only neck-foreleg components appeared and seldom was the whole gamut of movements run at any one time. When it was, the sequence was always the same: (1) lateral movement of the head with forward movement of the arm, (2) rotation of the head, neck, and forward part of the trunk, (3) flexion of lower trunk, and (4) flexion or abduction of the hind limb at the hip. When components were omitted they were usually the ones most recently acquired.

Stimulation by means of a probe, needle, brush, or glass rod resulted in the appearance of a number of movements not seen spontaneously. Stimulation of the proximal end of the tail seemed to produce flexion of the tail in 25 mm. fetuses; it could not be observed spontaneously in any smaller than 28 mm. long. Rippling of the

TABLE IV

SUMMARY OF MOTOR RESPONSES OF 21 MM.–30 MM. CAT FETUSES, ELICITED BY STIMULATION OR APPEARING SPONTANEOUSLY*

Line No.	Index No. in Table I	Litter No.	Embryo No.	Size in mm.	Neck and Trunk				Fore-Limb Movement				Hind-Limb Movement			Head	
					Head Flexion and Rotation	Trunk Flexion and Rotation	Tail Flexion	Abdominal Muscles	Shoulder	Elbow	Wrist	Digits	Hip	Knee	Ankle	Jaw	Tongue
91	32	85:31	1	21	+	+	–	–	+	+	+	–	+?	–	–	–	–
92	32	85:31	2	21	+	+	–	–	+	+	+	–	+	–	–	–	–
93	32	85:31	3	21	+	+	–	–	+	+	+	–	?	–	–	–	–
94	32	85:31	4	21	+	+	–	–	+	+	+	–	+?	–	–	–	–
95	33	20:32	2	21	+	+	–	–	+	+	+	–	+	–	–	–	–
96	33	20:32	1	22	+	+	–	–	+	+	+	–	+	–	–	–	–
97	33	20:32	3	23	+	+	–	–	+	+	+	–	+	–	–	–	–
98	34	14:32	1	25	+	+	+?	+	+	+	+	–	+	–	–	–	–
99	34	14:32	2	25	+	+	+?	+	+	+	+	–	+	–	–	–	–
100	34	14:32	3	25	+	+	?	+	+	+	+	–	+	–	–	–	–
101	35	90:31	4	26	+	+	+	?	+	+	+	–	+	–	–	–	–
102	35	90:31	1	28	+	+	+	?	+	+	+	–	+	–	–	–	–
103	35	90:31	2	28	+	+	+	?	+	+	+	–	+	–	–	?	–
104	35	90:31	3	28	+	+	+	?	+	+	+	–	+	–	–	–	–
105	36	19:32	2	28	+	+	+	+	+	+	+	–	+	+	–	+	+
106	36	19:32	1	30	+	+	+	+	+	+	+	–	+	+	–	+	+
107	36	19:32	3	30	+	+	+	+	+	+	+	–	+	+	–	+	+
108	36	19:32	4	30	+	+	+	+	+	+	+	–	+	+	–	+	+
109	37	95:31	1	30	+	+	+	+	+	+	+	–	+	+	–	+	+
110	37	95:31	2	30	+	+	+	+	+	+	+	–	+	+?	–	+	+
111	37	95:31	3	30	+	+	+	+	+	+	+	–	+	?	–	+	+
112	37	95:31	4	30	+	+	+	+	+	+	+	–	+	+	–	+	+
113	38	17:32	1	30	+	+	+	+	+	+	+	–	+	+	–	+	+
114	38	17:32	2	30	+	+	+	?	+	+	+	–	+	+	–	+	+
115	38	17:32	3	30	+	+	+	?	+	+	+	–	+	+	–	+	+
116	38	17:32	4	30	+	+	+	?	+	+	+	–	+	+	–	+	+
117	38	17:32	5	30	+	+	+	?	+	+	+	–	+	+	–	+	+
118	38	17:32	6	30	+	+	+	?	+	+	+	–	+	+	–	+	+
119	39	28:32	1	30	+	+	+	+	+	+	+	–	+	+	–	+	+
120	39	28:32	2	30	+	+	+	+	+	+	+	–	+	+	–	+	+
121	39	28:32	3	30	+	+	+	+	+	+	+	–	+	+	–	+	+
122	39	28:32	4	30	+	+	+	+	+	+	+	–	+	+	–	+	+

abdominal wall followed brushing or pressing upon the abdomen in 25 mm. embryos. This contraction was not observed spontaneously in any specimens of the present group. Simple flexion of the lower part of the trunk sometimes followed tapping of the hip region. Isolated movements of the forelimb could be induced in most of the specimens. These were similar to the ones observed in smaller embryos. Often, extension at the elbow and flexion at the wrist resulted from stimulation before it appeared spontaneously. Similarly, knee-bending could be induced. Touching the nose or ears resulted in a quick backward jerk of the head in most of the specimens.

In the smaller fetuses (21 mm.–25 mm.), many of the isolated induced movements were not followed by any generalized activity. In some cases, a local twitch of the foreleg or a quick contraction of abdominal muscles did serve to initiate a descending wave of the generalized writhing motility. This was usually the case in the 30 mm. fetuses. The quick jerk of the head was always followed by generalized mass-reactions. Stimulation of the foreleg never produced movements in the lower part of the body independent of the writhing mass movements; it did evoke a response in the opposite foreleg, at times. Stimulation of one hind leg was not infrequently followed by: (1) a local contraction at the knee and hip, (2) a quick contraction of the lower trunk with flexion of the opposite hind leg, (3) flexion of the opposite foreleg, and then (4) a generalized wave of writhing movements. Sometimes a quick jerk of the head followed the foreleg flexion and preceded the writhing movements.

These experiments indicate, again, that clearly defined local contractions make use of muscles not yet involved in the purely spontaneous activity. They also show that conduction of impulses rostrad precedes that caudad.

THIRTY-ONE TO THIRTY-SIX DAYS

Spontaneous movements were more active in fetuses 34 mm.–45 mm. long than they were in smaller ones. They involved nearly all muscles; eye and facial movements, as well as flexion of the digits of the forelegs, were seen occasionally in the 40 mm. specimens. The generalized undulating or writhing movements observed in earlier fetuses were even more pronounced; the rotation component was

more extensive. During the present period, movements of the foreleg and, to a smaller extent, those of the head begin to separate from the mass-motility. Independent spontaneous foreleg and jaw movements were frequently observed.

Induced contractions were likewise more marked than in smaller fetuses. All the movements seen at one time or another spontaneously, as well as some others which did not occur spontaneously, could be elicited by stimulation. Many of them seemed to show a purposeful character. Thus, touching the nose with the point of a needle almost invariably resulted in a quick jerk of the head away from the point of stimulation. Similarly, the legs were usually drawn up when the feet were pinched lightly; an extension was often seen in the opposite leg. When the tail was stimulated it was flexed between the hind legs. Stimulation of the ears was sometimes followed by a forward movement of the forelegs—the fetus appeared to be warding off a blow.

The transmission of impulses could be followed quite distinctly in a number of 38 mm. and 40 mm. fetuses. Stimulation of one hind foot, producing flexion at the knee and hip on that side, preceded the following series of events: (1) flexion of opposite hind leg (rarely, if ever, extension) at the knee and hip accompanied by quick flexion of lower trunk, (2) extension (straightening) of trunk, (3) extension of both forelegs (sometimes flexion preceded this extension), (4) quick extension of the head, and (5), as though released by the head-jerk, a descending wave of writhing movements. Stimulation of one forefoot, producing local flexion at the wrist, elbow, and shoulder, was followed by: (1) extension of the opposite leg at the elbow and shoulder, (2) quick extension of the head and neck, and (3) a descending wave of writhing movements. Stimulation of the nose was followed immediately by the head jerk and then writhing activity—not by simple reflexes in the fore and hind legs. Never was foreleg stimulation followed by any hind leg reflexes except those that were part of the secondary descending wave of generalized squirming movements.

Of course, many attempts to observe distinct reflexes were rendered unsuccessful by the active mass reactions described as writhing movements. More often than not, a spasm of activity was super-

imposed on local reflexes so that it was very difficult to be certain as to what was a simple reflex and what was mass-reaction. Careful localization of the stimulus applied to a hind foot was necessary in order to avoid disturbing the fetus before the nicely timed, ascending chain of reflexes could be observed. Even when this was accomplished, the initial local response often sufficed to move the fetus passively; consequently, unintentional, secondary stimulation of the head occurred and writhing activity appeared before more rostral, referred reflexes of the chain could be seen. Nevertheless, a number of positive results show that ascending waves of reflex behavior do precede the predominant descending ones.

DISCUSSION

At the time of a previous communication (Windle and Griffin, 1931) we held the opinion that the earliest movement of the cat embryo is ventrolateral head-flexion, produced by neck muscles which very soon bring about a passive forward flexion of the forelimb as part of a mass reaction. The present study seems to call this statement into question. It should carry more weight than the previous one because nearly five times as many specimens of the critical stages between 12 mm. and 18.5 mm. long have received attention. Furthermore, the technical procedures used have been much superior to those of the first experiments. In spite of the small amount of data and inferior methods, the observations of *spontaneous* movements that were made previously are in very close agreement with the new ones. A greater number of the present embryos between 16 mm. and 18.5 mm. long exhibit head movements than show foreleg flexion; this was exactly the case of the embryos of similar size in the former study where no motility was encountered before the beginning of the twenty-fifth day (16 mm.). The chief difference between the two groups of experiments lies in the fact that definite spontaneous motility has been observed in a number of younger embryos of the new series. The very early movements are more prominent in the forelimb than in the neck. The explanation of this may be that a very feeble muscular contraction can manifest itself mechanically with greater ease in a light mass, such as the limb-bud, than in a heavier one, such as the head. Granting that this

is true, we still must alter the former statement and say that the early spontaneous movements appear in the foreleg and neck muscles of cat embryos 14.5 mm.–15.5 mm. long between the twenty-fourth and twenty-fifth day after insemination.

Few detailed studies of the behavior of young embryos of comparable sizes have been reported. Tuge (1931) found that turtle embryos move spontaneously before stimulation elicits reflex responses. Movements are total reactions of the head, neck, and trunk; later the limbs move as part of a total behavior pattern. Kuo (1932) has observed that head movements appear first in chick embryos at the end of the third day; these are purely spontaneous. Angulo (1932) has just published observations on rat embryos. In general agreement with our previous study on the cat (Windle and Griffin, 1931) and that of Swenson (1928 and 1929) on the rat he found that lateral head flexion appears first; it was elicited by touching the snout with a hair. Spontaneous movements are rare in the early rat embryo and no forelimb movements occur at the time of first appearance of reflexes. Coghill (1909 and 1929) determined that the first movement in the larval amphibian is a simple lateral flexion of the cephalic part of the trunk. It is evident that our new observations on the priority of spontaneous motility in the fore limb over that of the head of the cat embryo are at variance with these. In seeking a reason for this difference between ours and other studies we might suggest that the behavior pattern of the cat never recapitulates all the stages seen in more primitive forms. May this pattern not begin as a mass movement which already involves the shoulder-girdle musculature and is apparent only in the lighter limb-bud? This explanation alone would be adequate if our observations were confined to spontaneous behavior, but the study of movements resulting from stimulation of the embryos raises questions of great significance.

Can we adhere to the conception that somatic motility develops from a total movement, fully integrated from the start, when simple contractions involving isolated muscle groups can be elicited before any mass-reaction appears? We see nothing adverse to such a theory if the movements elicited are the result of direct stimulation of muscle. If they result from stimulation of sense organs in epithelium or

in muscle, and are, therefore, reflexes, we must seek other interpretations. It is pertinent to inquire first of all whether they are active or passive. There is no doubt in the minds of any who have observed our experiments on 13 mm.–15 mm. embryos that stimulation often produces a slight active local twitch, not to be confused with any passive rebound phenomenon. Comparison of litters 32:32 and 33:32 (p. 528) shows this most strikingly. Although none could be elicited in neck muscles of any smaller than 14 mm. long, a number of 13 mm. embryos and one of 12 mm. exhibited foreleg movements in reponse to pressure stimuli. Are these active movements true reflexes involving a nervous mechanism or do they result from direct stimulation of muscles as yet unconnected with the nervous system? It is impossible to determine this satisfactorily at present. It would be difficult, indeed, to prove that they do not result from the transmission of impulses from the point of stimulation to the spinal cord and back to the muscle. The fact that a slight, but grossly appreciable, latent period usually ensued after stimulation and that summation of stimuli was often necessary before the response was invoked, makes it seem possible that a nervous mechanism is involved. Furthermore, we know that the spinal nerves and spinal cord are remarkably well developed just before the time movements are elicitable and that sensory and motor elements are being integrated by the ingrowth of sensory collaterals at that time (Windle, 1931 and 1933). Miss East (1931) believes that the formation of nerve endings may be the essential precursor of movement in the albino rat. She observed the beginning development of terminations on skeletal muscles of the forelimb on the day that the first movements can be elicited. The possibility that some of the early motor responses are neurogenic cannot be ignored.

On the other hand, these arguments do not prove that the early contractions of muscle are neurogenic. That some contractions may be myogenic might be inferred from the fact that they are often well localized at a time when the intrasegmental structure of the nervous system is diffuse. Furthermore, the apparent summation of stimuli necessary to elicit them should not be accepted too readily; although responses did not often follow the application of a single pressure stimulus, their appearance after a series of stimuli may involve a

factor of coincidence. Finally, the presence of a few sensory collaterals in the nervous system of some embryos of comparable ages does not mean that these structures have developed in the early specimens of the present series. We have examined few of them histologically. Premotile embryos (11 mm.) of litter 3:32 do possess a few collaterals which pass from the posterior funiculus into the alar plate among secondary sensory neuroblasts (Windle, 1933). It is hoped that further studies will clarify the nature of early motor responses.

SUMMARY

Spontaneous movements were observed in cat embryos 14.5 mm.–15 mm. long, first in the fore limb, then in the neck. The development of mass-movements of the neck, trunk, and limbs is totally integrated from the beginning and is believed to be traceable to these early spontaneous flexions. More detailed motility becomes distinct from the primitive massive reactions at a later time. The gradual cephalocaudal expansion of trunk-limb motility results in squirming or writhing activities which are thought to precede the appearance of locomotor mechanisms. Simple, local unit muscular contractions can be elicited a day earlier than spontaneous mass movements. These unit contractions, if not actually neurogenic, involve musculature which is later employed by reflexes. Carefully localized stimulation of small cat fetuses elicits isolated movements locally and reflex contractions of muscles in regions rostrad. However, reflexes farther caudad never ensue until after the expanding, massive, total pattern (locomotor reaction) has extended below the points stimulated.

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THE LONGITUDINAL GRADIENT IN STYLOCHUS IJIMAI WITH A CRITICAL DISCUSSION

(Thirty-two figures)

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THE pr[redacted]nt paper is a study of the longitudinal gradient in the [redacted]yclad, *Stylochus ijimai* Yeri et Kaburaki.[1] It represents [redacted]part of the work done by the Rockefeller Foundation visiting profess[redacted] of 1930–31 at Tohoku Imperial University and his assistant. Most of the data were obtained during July and August, 1931, at the Marine Biological Laboratory of Tohoku Imperial University near Asamushi. To Dr. S. Hatai, Director of the Station and to other members of the staff, acknowledgment is due for the laboratory facilities provided and for many personal kindnesses.

For the data on CO_2 production Watanabe is responsible. The supplementary data on the indophenol gradient and the developmental changes in the susceptibility gradient were obtained by Child.

I. THE RESPIRATORY GRADIENT: CO_2 PRODUCTION

MATERIAL

The worms were collected from a sand flat at Asadokoro, a small fishing village near Kominato railway station on the Tohoku line, Aomori-Ken, in summer. They varied in length from 8 to 30 mm. and almost all individuals collected showed some stage of the reproductive cycle, most of them some stage in the enlargement of the ducts by the accumulation of spermatozoa or eggs. Differences in stage of the reproductive cycle in different individuals made it possible to determine CO_2 production in three stages which were distinguished as follows: Stage I, genital atrium and copulatory organ visible in ventral view of living animal but ducts not yet distended with sexual

[1] The authors are much indebted to T. Kaburaki, Professor of Zoölogy in the Agricultural Department of Tokyo Imperial University, who identified the species. Specific morphological characters are described by Yeri and Kaburaki (1918, pp. 6–8).

products; Stage II, posterior part of oviducts distended by eggs approximately to the level of the posterior end of the pharynx; Stage III, oviducts distended by eggs over whole length.

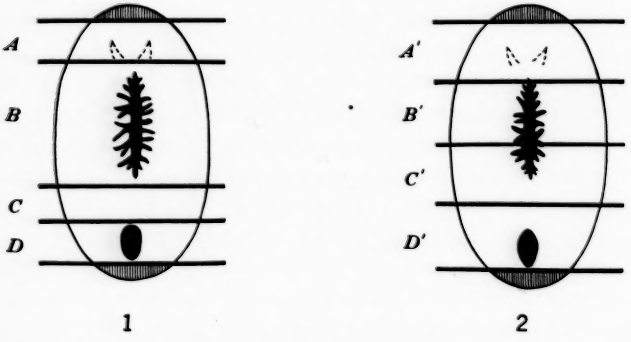
METHODS

For the determination of the rate of CO_2 production Parker's modification of Osterhout's indicator method (Parker, 1925) was employed. The time required for the change of hydrogen-ion concentration from pH 7.78 to 7.36 was determined. The respiratory chambers used were small Erlenmeyer flasks of pyrex glass with a tube fused into the side of the flask just above the bottom in such position that when the flask is upright the tube extends horizontally. This tube opens into the respiratory chamber at one end and is closed at the other. It is about 1.5 cm. in diameter, i.e., the same diameter as the standard indicator tubes used and serves for comparison of the indicator solution with the standards. The total volumes of 4 respiratory chambers including the side tube are respectively 30.5 cc., 30.8 cc., 31.6 cc., and 32.2 cc. The volume of the tube is about 9 cc. The standard solution was made of boric acid and borax according to the method of Palitzsch (Kolthoff and Furman, 1926, p. 145) and phenol red in saturated water solution was added as indicator. The individuals or pieces were wrapped in a small piece of thin, rough gauze and tied on a small glass rod attached to the stopper in such position that they were approximately in the center of the air space of the chamber. Ten cc. of indicator solution in 0.0001 M $NaHCO_3$ were placed in the bottom of the chamber, the air of the chamber replaced by CO_2-free air, and the chamber closed by the stopper with the material attached. The chamber was then placed on a tilting apparatus which changed its position 30 times a minute in such manner that the indicator solution ran back and forth between the flask and the tube, but without violent shaking.

The volume of the animal or pieces plus the gauze may be regarded as negligible, for even if it is as great as 0.5 cc., and in no case has it been more than that, the error resulting from neglecting it is only 1.5 per cent. The calculation of CO_2 was made according to Parker's formula (Parker, 1925).

The worms used for experiment were kept without food for 5 or

10 days after collection in order to minimize differences in digestive activity. CO_2 determinations were made on intact worms. Animals were cut transversely into 2 pieces for determination of the effect of section on CO_2 production, and in the determination of CO_2 production at different levels the body was cut into 3 or 4 pieces excluding extreme anterior and posterior ends which were discarded. In most of the determinations on different body levels the animals were cut into 4 pieces, *A*, *B*, *C*, *D* as indicated in Figure 1. Piece *A* is



FIGS. 1 and 2.—Diagrammatic outlines indicating pieces used for CO_2 determinations: the shaded anterior and posterior ends were discarded. Fig. 1, *A–D*, the pharyngeal piece, *B*, is longer than the others leaving the pharynx intact. Fig. 2, *A'–D'*, four pieces of equal length.

prepharyngeal and contains the cephalic ganglion; piece *B* is the pharyngeal region; piece *C* includes the region between the pharynx and genital atrium; piece *D* is the posterior piece which contains the terminal sex organs. In all cases the extreme anterior and posterior regions indicated by shading in Figure 1 were discarded.

In certain experiments in which repeated determinations were made on the same pieces it was found better to use 3 instead of 4 pieces on account of a peculiar reaction of the species. The body tissues of the living animal are very tough and offer considerable resistance to the knife and to pressure, but after repeated cutting or sometimes even after the repeated handling necessary in repeated CO_2 determinations, pieces or mutilated animals undergo disintegration within a few seconds. The body entirely loses its firmness and

structure and "melts" into a soft mass of débris. This disintegration can also be induced by local chemical stimulation of some part of the body. In the repeated CO_2 determinations on the same pieces it was found that such disintegration sometimes occurred, particularly in the short *C*- and *D*-pieces. Consequently three pieces *A*, *B*, and *C*+*D* (Fig. 1) were used.

With the method of section shown in Figure 1 the piece *B* is much longer than the other pieces. In order to determine whether factors associated with this difference in length of the different pieces were concerned in the differences in CO_2 production which were found at different levels, another series of experiments was performed in which the body, except the extreme anterior and posterior ends, was divided into four pieces of approximately equal length (*A'*–*D'*, Fig. 2).

Stylochus is able to live without injury for a long period in saturated air. Both in water and saturated air it may remain without appreciable movement for many hours. Also it does not secrete a large quantity of mucus when stimulated or cut, consequently accurate weighing is less difficult than in the case of *Planaria*, for example. Of the four pieces *A*, *B*, *C*, *D*, the piece *A* which contains the cephalic ganglia shows more motor activity after section than the other pieces and is capable of co-ordinated, progressive locomotion like the intact animal. This activity decreases after section and if undisturbed the piece gradually becomes quiet. The postcephalic pieces are much less active, show very incomplete co-ordination, and soon become quiet after section.

All data presented in the following tables, except those determined immediately, thirty minutes, and 1 hour after section, are average values obtained from 2 successive determinations.

DATA ON CARBON DIOXIDE PRODUCTION

Effect of section on the rate of CO_2 production.—As Hyman (1923) has shown for *Planaria*, the rate of respiration is temporarily increased by section. In any attempt to determine the differences in respiration at different body levels in isolated pieces, it is necessary first to investigate the effect of section. Data concerning this point are given in Tables I, II, and III.

In Table I the effect of sectioning on individual worms is shown. In this experiment, after determination of CO_2 production in intact

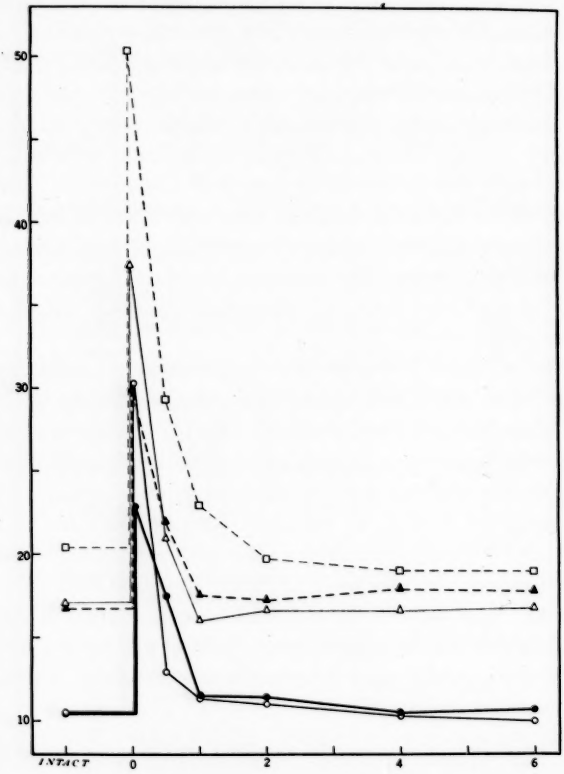


FIG. 3.—Effect of section on CO_2 production: animals separated into two pieces by transverse cut at posterior end of pharynx (between *B* and *C*, Fig. 1): plotted from data of Table I. Ordinates, thousandths of a milligram CO_2 per gram per minute; abscissae, hours after section. The curves of the different animals of Table I are indicated as follows: No. 1, solid black circles; No. 2, circular outlines; No. 3, solid black triangles; No. 4, triangular outlines; No. 5, squares.

animals, the animals were cut into 2 parts, $A+B$, and $C+D$, without removal of extreme anterior and posterior regions of the body and CO_2 determinations on both pieces together were made again at

different times as follows: immediately, 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours after section. The intact worms were weighed after the first determination, and pieces, after the seventh determination. During the intervals between determinations worms and pieces were kept in sea water.

Table I and Figure 3 show that the rate of CO_2 production by worms is abruptly increased by sectioning, the increase ranging from

TABLE I

EFFECT OF SECTION ON THE RATE OF CO_2 PRODUCTION IN *Stylochus ijimai*, STAGE II

CO_2 in Mgm. per Gm. per Min. In each test one worm in Stage II was used.

No. of Test	1	2	3	4	5	Average
Temperature range in degrees C.	17.8–18.3	16.8–18.0	18.1–18.5	17.2–17.6	18.2–19.0	16.8–19.0
Intact worm	0.0104	0.0105	0.0167	0.0170	0.0203	0.0150
Immediately after section	0.0228	0.0303	0.0299	0.0373	0.0504	0.0341
½ hour	0.0174	0.0129	0.0219	0.0209	0.0293	0.0205
1 hour	0.0114	0.0113	0.0175	0.0160	0.0226	0.0158
2 hours	0.0114	0.0110	0.0172	0.0166	0.0197	0.0152
4 hours	0.0105	0.0103	0.0179	0.0166	0.0190	0.0149
6 hours	0.0107	0.0100	0.0173	0.0163	0.0190	0.0147

79 to 188 per cent over the rate for intact animals. A rapid decrease follows during the first hour after section, then a gradual further decrease, finally reaching comparatively constant, and approximately the same values as those observed in intact worms.

Similar changes in rate of CO_2 production following section can be seen in isolated pieces representing a particular region of the body. Table II and Figure 4 show it for *B* pieces of Stage II. CO_2 determinations on *B* pieces were carried on at different times after isolation of the pieces, that is, immediately, 30 minutes, 1 hour, 2 hours, and each following hour up to 5 hours. At the end of 6 hours another cut was made in order to determine whether another increase

in CO_2 production would occur. This second cut was longitudinal along the course of the oviduct lateral to the pharynx on one side, and separated the pieces into 2 unequal parts. Following this second cut CO_2 determinations were made immediately, 30 minutes, 1 hour, 2 hours, and 3 hours after the second section. Pieces were weighed after the third determination, and this weight was taken as the weight for the first seven tests. A second weighing was made after the tenth determination and this weight was taken as that for the determinations after the second section. Pieces were kept in sea water during each interval between determinations.

Figure 4 is a graph of the data in Table II. The graph shows that the rate of CO_2 production is highest immediately after isolation from the body and decreases rapidly during the first hour and later very gradually. But immediately after the second section, CO_2 production increases again abruptly and this increase is also followed by a rapid decrease so that 1 hour after the second section the rate is approximately the same as that 1 hour after the first section and the further decrease is very slow. These data in Tables I and II and Figures 3 and 4 indicate that the gush of CO_2 following section is an expression of the stimulation resulting from sectioning. As this stimulation decreases, CO_2 production also decreases, approaching a constant value, which as Table I and Figure 3 show is the rate of intact animals before section. From these results, it is evident that in order to obtain a comparatively constant and approximately normal rate of CO_2 production in pieces determinations should not be made until at least one hour after section.

CO_2 production of pieces from different body levels.—The preceding data tell us nothing concerning the effect of section on pieces from different levels of body. Pieces from different levels may be affected in very different degrees by section and the effect may persist longer in some pieces than in others. Consequently, the differences in CO_2 production immediately or soon after section may be very different from those in the intact unstimulated animals. The only way in which further light can be thrown on this question is by CO_2 determinations on pieces from different body levels at different periods after section. Such determinations are given in Table III. For these experiments worms of Stage I were cut into 3 pieces A, B, and $C+D$,

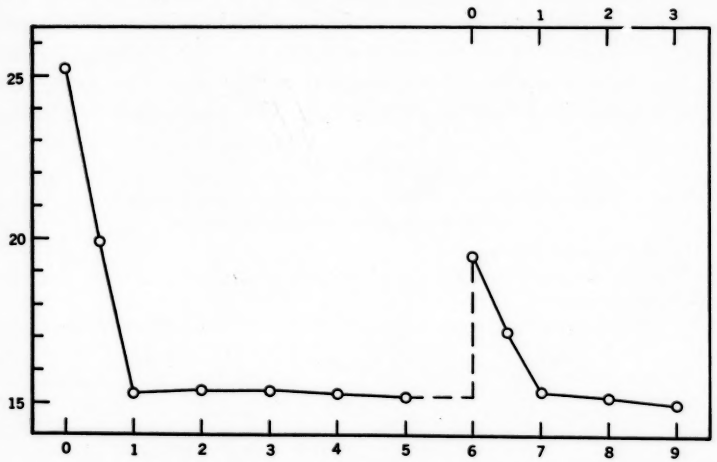


FIG. 4.—Effect of section on CO_2 production in *B* pieces: plotted from data of Table II. ordinates, thousandths of a milligram CO_2 per gram per minute; abscissae, hours after section. CO_2 production decreases rapidly during first hour after section to almost constant rate. Second section 6 hours after first brings about second increase but only about half as great as first, and also followed by a rapid decrease.

TABLE II

EFFECT OF SECTION ON THE RATE OF CO_2 PRODUCTION FROM PIECES OF *Stylochus ijimai*

In each determination two *B* pieces from animals in Stage II were used.

No. of Test	Hours after 1st Section	Hours after 2d Section	Temperature in Degrees C.	CO_2 in Mgm. per Gm. per Min.
1	0	.....	20.0	0.0252
2	½	.....	20.3	0.0199
3	1	.....	20.3	0.0153
4	2	.....	20.5	0.0154
5	3	.....	21.0	0.0154
6	4	.....	21.0	0.0153
7	5	.....	20.8	0.0152
8	6	0	20.5	0.0195
9	6½	½	20.0	0.0172
10	7	1	20.0	0.0154
11	8	2	19.5	0.0152
12	9	3	19.5	0.0150

and CO_2-determinations were made on all 3 lots immediately and every 2 hours after section up to 6 hours.

The data of Table III are graphed in Figure 5. The table and graph show the following points: first, CO_2 production is greatly

TABLE III

CO_2 PRODUCTION IN PIECES OF *Stylochus ijimai*, STAGE I, FROM DIFFERENT LEVELS OF BODY

CO_2 in Mgm. per Gm. per Min.

Determinations were made every two hours after section up to six hours. Each lot consists of three pieces.

No. of Test	Temperature Range in Degrees C.	Intact Worms	Hours after Section	Pieces		
				A	B	C+D
1.....	18.4–18.8	0.0130	0	0.0448	0.0221	0.0406
			2	0.0189	0.0100	0.0139
			4	0.0183	0.0099	0.0140
			6	0.0180	0.0097	0.0137
2.....	18.0–18.6	0.0161	0	0.0506	0.0223	0.0546
			2	0.0183	0.0155	0.0143
			4	0.0183	0.0151	0.0140
			6	0.0176	0.0148	0.0138
3.....	18.4–19.2	0.0181	0	0.0287	0.0334	0.0556
			2	0.0152	0.0084	0.0173
			4	0.0145	0.0082	0.0159
			6	0.0149	0.0079	0.0159
4.....	18.6–19.3	0.0200	0	0.0332	0.0246	0.0245
			2	0.0192	0.0127	0.0168
			4	0.0182	0.0120	0.0166
			6	0.0180	0.0118	0.0168
5.....	18.3–18.8	0.0211	0	0.0342	0.0508	0.0440
			2	0.0147	0.0134	0.0223
			4	0.0145	0.0132	0.0220
			6	0.0147	0.0132	0.0217

increased by section, as in the preceding experiments; second, different lots immediately after section show very different gradient forms, U-shaped, inverted U-shaped, single with anterior high, single with posterior high; third, the gradient immediately after section may differ greatly from that in the same lot of pieces later, fourth, in later determinations, shown in the lower curves, the form

of gradient does not change and the values for each piece remain almost constant.

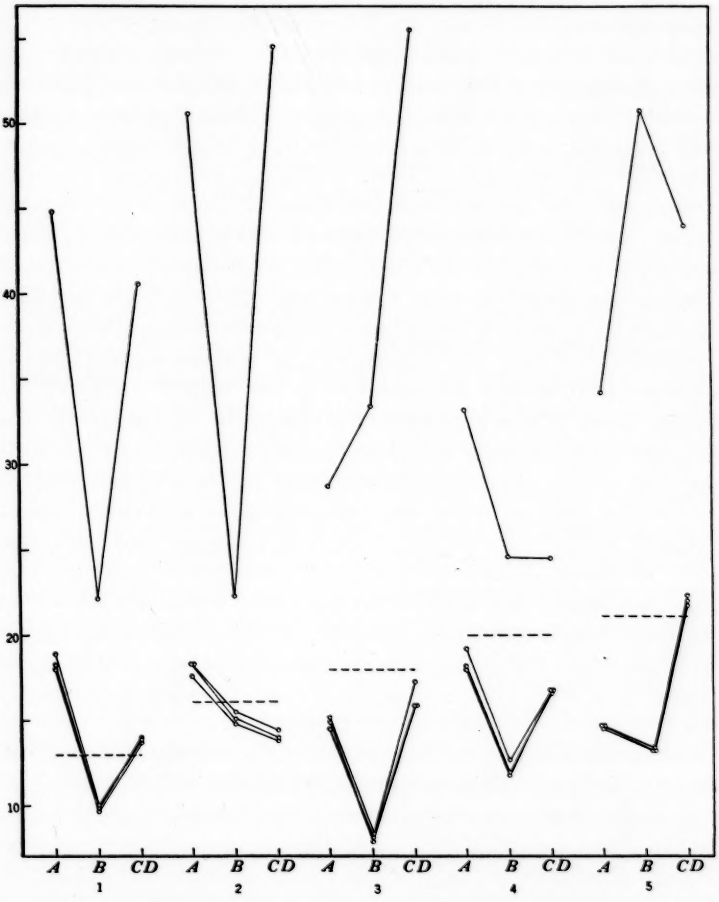


FIG. 5.—CO_2 production in pieces from three levels, *A*, *B*, *C*+*D*, Fig. 1, plotted from data of Table III. Ordinates, thousandths of a milligram CO_2 per gram per minute; abscissae, body levels, *A*, *B*, *C*+*D* (Fig. 1), each represented by 3 pieces in each of the 5 series of determinations. The upper curves of each series show CO_2 production immediately after section, the 3 lower curves, 2, 4, and 6 hours after section.

In 4 of the 5 lots the double gradient appears in the later determinations, but in the second lot the rate decreases from anterior to posterior, although this lot shows a double gradient immediately after section. The reason for this difference is not known. The double gradient with anterior and posterior pieces showing the high rates appears to be the characteristic form, but different lots show considerable variation in both relative and absolute rates of anterior and posterior pieces which may be due to incidental factors, such as motor activity of anterior pieces, in different individuals. In lots 1 and 2, the CO_2 production of the anterior pieces is considerably higher than that of the intact animals which is indicated by the horizontal broken line. In lots 3 and 4 all pieces and in lot 5 anterior and middle pieces show a lower rate than the intact animals. As regards the wide variation immediately after section the data are similar to the results of previous work by Watanabe (1931) on the axial gradient of CO_2 production in an earthworm, *Allolobophora foetiḍa* (Sav.). There also wide variation in the rate of pieces from different regions of body occurs and a definite form of axial gradient does not appear at once after isolation of pieces, but, as time goes on and the effect of section decreases, a distinct U-shaped gradient becomes evident. According to Hyman's observations (Hyman, 1932) on *Nereis*, differences in rate of oxygen consumption based on the data obtained during the first 3 hours after separation into pieces are also different from those based on the data obtained during the second 3 hours, the latter showing a distinct double gradient. As these experiments show, unless the CO_2 or O_2 determinations are made after the effect of section has disappeared or at least decreased to a considerable degree, the results of the experiments may not give any significant data concerning the gradients of animals.

Various factors are probably concerned in this wide variation during the period soon after section. In the first place, the method used requires a considerable time for the change in color of the indicator solution and during this time the CO_2 production of the pieces is undergoing rapid decrease, probably at different rates in pieces from different levels and from different individuals. If it were possible to make determinations in a few seconds or even in a few minutes we might find a definite characteristic gradient immediately after sec-

tion, although it might differ in form from that found later. At present, however, it is impossible to obtain any evidence on this point because available methods are inadequate for the purpose.

CO_2 production in animals of different stages of sexual development. —In the attempt to avoid as far as possible the great increase, rapid decrease, and wide variation in CO_2 production following section which is indicated in the preceding data, CO_2 determinations in this series of experiments were begun $2\frac{1}{2}$–3 hours after section. Two

TABLE IV

AXIAL GRADIENT OF CO_2 PRODUCTION IN PIECES A–D OF *Stylochus ijmai*, STAGE I

Each lot consists of six pieces.

No. of Test	Temperature, Degrees C.	CO_2 in Mgm. per Gm. per Min.			
		A	B	C	D
1	21.2–21.4	0.0135	0.0106	0.0123	0.0178
2	24.5–25.0	0.0164	0.0132	0.0147	0.0163
3	25.3–26.1	0.0200	0.0110	0.0172	0.0239
4	24.0–24.3	0.0208	0.0146	0.0237	0.0275
5	24.0–24.6	0.0210	0.0125	0.0163	0.0219
6	22.3–22.7	0.0250	0.0185	0.0166	0.0252
7	24.3–24.6	0.0262	0.0099	0.0135	0.0180
Average		0.0204	0.0129	0.0163	0.0215

determinations were made successively on each lot and the tabulated rate is the average of these two determinations. Table IV gives data for Stage I, Table V for Stage II, Table VI for Stage III. The animals of Stage I were 1–1.8 cm. in length and each experimental lot consisted of 6 pieces. The animals of stages II and III were 2.2–3.0 cm. in length and each lot consisted of three pieces. Figure 6 is graphed from the average of Tables IV to VI.

In Figure 6 the medium heavy line shows the gradient in Stage I, the heavy line that of Stage II, and the light line that of Stage III. The gradient of Stage I is essentially similar in form to that of the 4 lots of Table III and the curve of Figure 5, 2 hours and later after section. Also Table IV shows that in 6 of the 7 lots the data give essentially the same form of curve. Only No. 6 differs from the

others in that the *C* pieces show a slightly lower rate than the *B* pieces, but the general form of gradient is the same as in other lots.

TABLE V

AXIAL GRADIENT OF CO_2 PRODUCTION IN PIECES A–D OF *Stylochus ijimai*, STAGE II

Each lot consists of three pieces.

No. of Test	Temperature, Degrees C.	CO_2 in Mgm. per Gm. per Min.			
		A	B	C	D
1	22.0–22.2	0.0136	0.0153	0.0170	0.0225
2	20.5–20.8	0.0141	0.0102	0.0234	0.0192
3	21.2–22.0	0.0141	0.0164	0.0180	0.0183
4	21.0–21.3	0.0155	0.0190	0.0199	0.0163
5	21.3–21.6	0.0182	0.0154	0.0244	0.0214
6	24.5–25.0	0.0183	0.0165	0.0193	0.0182
7	21.8–22.2	0.0195	0.0182	0.0300	0.0253
8	21.2–21.5	0.0233	0.0149	0.0286	0.0269
Average		0.0171	0.0157	0.0226	0.0210

TABLE VI

AXIAL GRADIENT OF CO_2 PRODUCTION IN PIECES A–D OF *Stylochus ijimai*, STAGE III

Each lot consists of three pieces.

No. of Test	Temperature Degrees C.	CO_2 in Mgm. per Gm. per Min.			
		A	B	C	D
1	24.3–24.6	0.0116	0.0089	0.0104	0.0141
2	24.4–24.6	0.0143	0.0118	0.0216	0.0145
3	25.3–26.0	0.0155	0.0130	0.0196	0.0208
4	24.3–25.0	0.0164	0.0225	0.0180	0.0195
5	24.5–24.7	0.0175	0.0187	0.0170	0.0221
6	24.5–24.8	0.0194	0.0121	0.0150	0.0204
7	24.3–24.7	0.0200	0.0130	0.0164	0.0175
8	24.7–24.7	0.0227	0.0152	0.0172	0.0183
9	24.0–24.2	0.0258	0.0310	0.0207	0.0236
10	24.3–25.0	0.0260	0.0158	0.0112	0.0151
Average		0.0188	0.0162	0.0167	0.0186

In Stage II (Table V and heavy line in Fig. 6) the average CO_2 production decreases from *A* to *B*, increases to its highest point in *C*,

and again decreases somewhat again in *D*. The data of Table V give this form of curve with the highest rate in *C* in 5 of the 8 lots. In lots 1, 3, and 4 of Table V, the *B* pieces show a somewhat higher rate than the *A* pieces but not as high as that of the *C* pieces. In lots 1 and 3 the rate increases from *A* to *D*, that is, the gradient is single though some differences are only slight. The much higher rate of the *C* pieces in Stage II than in Stage I is probably associated with the fact that Stage II represents the period of rapid development and functional activity of the terminal reproductive organ-complex which is contained in the C pieces.

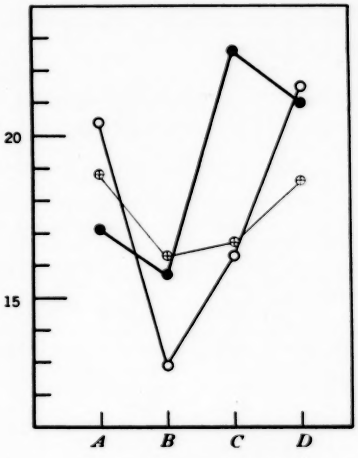


FIG. 6.—CO_2 production in pieces *A*–*D* of stages I–III. Plotted from averages of tables IV–VI. Ordinates, thousandths of a milligram CO_2 per gram per minute; abscissae, body levels of the pieces *A*, *B*, *C*, *D*. The curve of Stage I is marked by circular outlines, that of Stage II by solid black circles, that of stage III by circles with cross diameters.

In Stage III (Table VI and light line of Fig. 6) the average data give a U-shaped curve with *B* pieces at the lowest level but with little difference between *B* and *C* and much less steep than the curves of Stages I and II, but the data of the different lots of Table VI vary somewhat in the form of curve which they give. For example, in lots 4 and 9, the rate is highest in the *B* pieces and in lot 5 is somewhat higher in *B* than in *A*. In lot 2 the rate is highest in the *C* pieces as in Stage II. In lots 5 and 10 the rate is somewhat lower in *C* than in *B*. Among these variations the only cases which are distinctly anomalous are the high rates of the *B* pieces in lots 4 and 9. The differences between *B* and *C* are in general not great and the average difference is very small. It seems probable that differences in functional condition or activity of the reproductive organs, perhaps also differences in volumes of eggs or spermatozoa in the ducts, are the chief factors in determining

the observed differences in the different lots. It is also possible that the effects of section on parts of the reproductive complex differ in degree or rate of disappearance from those on the body in general, and that such differences are most evident in the fully developed organs of Stage III.

CO_2 production in pieces of equal length.—In the preceding experiments the *B* piece was made considerably longer than the other

TABLE VII

THE RATE OF CO_2 PRODUCTION IN PIECES OF EQUAL LENGTH FROM DIFFERENT LEVELS OF BODY OF *Stylochus ijimai*, STAGE I

CO_2 in Mgm. per Gm. per Min.
Each lot consists of three pieces.

No. of Test	Temperature Degrees C.	Intact Worms	Temperature Degrees C.	Pieces			
				A′	B′	C′	D′
1.....	16.5	0.0177	16.2–16.5	0.0155	0.0100	0.0147	0.0204
2.....	19.0	0.0181	19.1–19.1	0.0200	0.0153	0.0174	0.0251
3.....	16.9	0.0189	17.0–17.2	0.0179	0.0115	0.0153	0.0183
4.....	17.8	0.0206	18.0–18.3	0.0184	0.0134	0.0140	0.0254
5.....	18.9	0.0206	19.0–19.2	0.0184	0.0150	0.0155	0.0219
6.....	17.0	0.0208	17.0–17.2	0.0181	0.0124	0.0120	0.0223
7.....	16.7	0.0219	17.4–18.0	0.0224	0.0162	0.0169	0.0183
8.....	16.7	0.0236	17.5–18.0	0.0136	0.0133	0.0120	0.0259
Average	.....	0.0203	.....	0.0183	0.0134	0.0147	0.0222

pieces (see Fig. 1) in order that the pharynx might be left intact. But the possibility could not be excluded that the greater length of the *B* pieces might be associated in some way with their lower rate of CO_2 production. Therefore in order to avoid possible criticism on this point another series of experiments was performed with pieces of as nearly as possible equal length, animals of Stage I being used. The approximate regions represented by these pieces *A′*, *B′*, *C′*, *D′*, are indicated in Figure 2. As in other experiments the extreme anterior and posterior ends indicated by the shaded portions of Figure 2 were discarded. CO_2 determinations were made 2–3 hours after section on eight lots of 3 pieces each *A′*–*D′*. The data are given in Table VII and the averages are plotted in Figure 7. As Figure 7

shows, the resulting curve is U-shaped and very similar to the curve of Stage I in Figure 6. Moreover the data of the different series of Table VII show no important variations. In Nos. 6 and 8 the C' pieces show a very slightly lower rate than the B' pieces, and in No. 7 the rate of the A' pieces is somewhat higher than that of the D' pieces but the data of each series taken alone give the U-shaped curve. Evidently the results in the experiments with pieces of equal length are not essentially different from those in which the B pieces were longer. The low rate of the B pieces is not due to the fact that they are longer than other pieces.

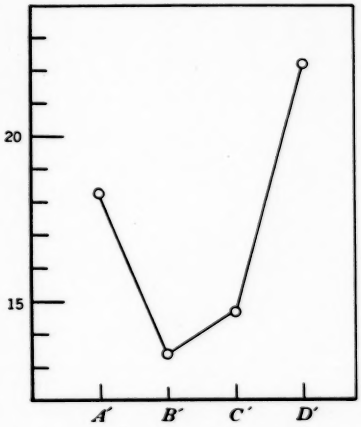


FIG. 7.—CO_2 production in pieces of equal length, A'–D', Figure 2. Plotted from data of Table VII. Ordinates thousandths of a milligram CO_2 per gram per minute; abscissae, body levels of the pieces, A'–D'.

CO_2 production of intact animals of stages I, II, and III.—For the purpose of comparison with data on respiration at different stages of the life-history in *Planaria* and other forms, CO_2 production of intact animals of stages I, II, and III was determined. Tables VIII–X give the data of those determinations. The averages, 0.0200 for Stage I (Table VIII), 0.0175 for Stage II (Table IX), 0.0107 for Stage III (Table X), show that the rate of CO_2 production decreases during the course of sexual development, i.e., with the progress of physiological senescence. This result agrees with the data of Hyman on oxygen consumption in relation to physiological age in *Planaria* (Hyman, 1919) and with the generally observed fact that respiratory metabolism decreases during the course of physiological senescence.

II. DEVELOPMENTAL CHANGES IN INDOPHENOL AND SUSCEPTIBILITY GRADIENTS

Embryonic and larval material—When the animals were kept in the laboratory for some days eggs were laid in large numbers and

developed normally. The eggs were attached to the surface of the container in a sheet consisting of a single layer of variable size and

TABLE VIII

CO_2 PRODUCTION OF INTACT ANIMALS, STAGE I

Each lot consists of one worm.

No. of Test	Temperature in Degrees C.	Body Weight in Gm.	CO_2 in Mgm. per Gm. per Min.
1	22.7	0.0734	0.0113
2	22.5	0.0906	0.0153
3	22.5	0.0499	0.0161
4	22.3	0.0669	0.0167
5	22.3	0.0757	0.0172
6	22.7	0.0755	0.0190
7	22.2	0.0395	0.0229
8	22.4	0.0710	0.0260
9	22.4	0.0727	0.0277
10	22.5	0.0516	0.0284
Average	22.5	0.0667	0.0200

TABLE IX

CO_2 PRODUCTION OF INTACT ANIMALS, STAGE II

Each lot consists of one worm.

No. of Test	Temperature in Degrees C.	Body Weight in Gm.	CO_2 in Mgm. per Gm. per Min.
1	21.7	0.0733	0.0110
2	22.0	0.0339	0.0147
3	21.5	0.0668	0.0155
4	21.7	0.0442	0.0158
5	21.7	0.0753	0.0165
6	21.5	0.1980	0.0170
7	21.5	0.1343	0.0195
8	21.6	0.0729	0.0200
9	21.9	0.0753	0.0212
10	21.7	0.1122	0.0235
Average	21.7	0.0886	0.0175

form, often a square centimeter or more in area. The early cleavages were visible through the membrane and it was evident that the eggs were not definitely oriented with respect to gravity. Movement be-

gan and continued for several days in the membrane and elongation of the larva and development of at least one of the "eyespots" took place before hatching. The Figures 8 and 9 show outlines of 2 stages of elongation which occur before hatching but which can readily be removed from the membranes with the aid of needles. The left eyespot usually if not always appears somewhat earlier than the right (Fig. 8) and the two are at first not symmetrical in position, the one on the right being somewhat farther anterior (Fig. 9). The larva is of

TABLE X

CO_2 PRODUCTION OF INTACT ANIMALS, STAGE III

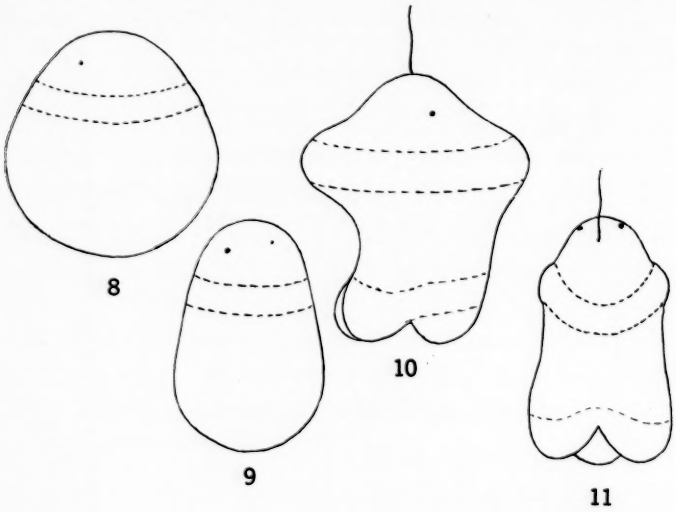
Each lot consists of one worm.

No. of Test	Temperature in Degrees C.	Body Weight in Gm.	CO_2 in Mgm. per Gm. per Min.
1	27.7	0.0781	0.0082
2	27.9	0.0998	0.0086
3	27.6	0.2457	0.0094
4	27.6	0.1390	0.0095
5	26.9	0.0995	0.0100
6	27.4	0.0950	0.0112
7	27.6	0.1370	0.0121
8	27.9	0.1553	0.0125
9	26.9	0.1420	0.0129
10	27.7	0.0781	0.0129
Average	27.5	0.1269	0.0107

the trochophore type with an apical flagellum, a strongly developed prototroch and a paratrochal band of cilia associated with 2 lobes which develop posteroventrally. Figure 10 shows a lateral, Figure 11 an anteroventral outline. The larvae lived for several days in the laboratory but showed no indications of metamorphosis and did not develop beyond the stage shown in the figures.

The axial differential in the indophenol reaction in embryonic and larval stages.—An axial differential in intracellular formation of indophenol has been demonstrated in various embryos and larval stages (Child, 1915, 1926) and in *Paramecium* (Child and Deviney, 1925) and various other unicellular forms. As is well known, this reaction consists in the formation of indophenol from α naphthol and dimethylparaphenylenediamine. Both of these substances penetrate

living cells readily and the intracellular reaction is catalyzed by oxidases. A differential or gradient in time of appearance and in amount of the blue particles of indophenol as estimated by appearance indicates the existence of a physiological gradient or differential of some sort in the cell or cell mass concerned. In employing this method on embryos and larvae of *Stylochus*, as in other cases, care must be tak-



FIGS. 8–11.—Developmental stages. Figures 8, 9, stages of elongation preceding hatching; the left "eyespot" usually develops earlier than the right; prototroch indicated by broken lines. Figure 10, lateral, Figure 11, anteroventral view of larva.

en to use very low concentrations of the two agents, particularly the α naphthol which is highly toxic. In sufficiently low concentrations all parts of the ectoderm may show the reaction and certain regions may become very deep blue while the larvae are still swimming actively, but in higher concentrations cytolysis may occur before the reaction. Below a certain limit the result does not depend on concentration except as regards time. For *Stylochus* material solutions were made as follows: a 1/2000–1/5000 solution of dimethylparaphenylenediamine hydrochloride in sea water and a solution from 0.3–1 mg. α naphthol in 3–5 cc. of sea water, but since this may not

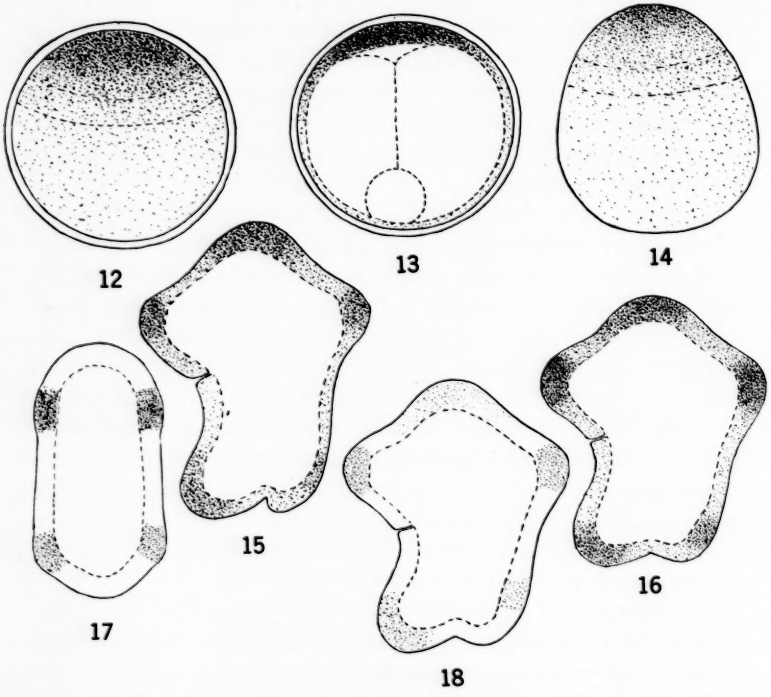
dissolve completely and is also very volatile the actual concentration of the solution is uncertain. These solutions were made fresh each day. One to several drops of each of these solutions was added to 3–5 cc. of sea water containing the animals. In some cases a very low concentration was used at the beginning and a drop or two of each solution was added from time to time, as observation of the material indicated: in other cases a higher concentration without addition was used. The indophenol differential may become evident in 10–15 minutes or only after one to several hours, according to concentration of agents.

In Figures 12–18 the attempt is made to indicate the indophenol differential by difference in depth of shading. Figure 12 is a surface view and Figures 13–18 are diagrammatic optical sections. The figures show the characteristic differences at different stages of development but do not show successive stages of the reaction in the same larva since to do that would increase greatly the number of figures. In Figures 12–18 the more deeply shaded parts indicate regions which are deep blue in the living animals; the parts least shaded, regions in which a diffuse blue is present of much lower concentration; and unshaded parts, regions in which the color has not appeared appreciably. The reaction was observed many times in hundreds of individuals and showed no appreciable variation in different individuals of the same developmental stage.

The results in all concentrations low enough so that death and cytolysis did not occur before the reaction and high enough so that a deep blue appeared within 3–4 hours were similar in all cases observed. The observations were made repeatedly on different lots of embryos with different concentrations. The amount of indophenol, as indicated by the color, decreased from the apical region basipetally in the ectoderm, the color gradient being very distinct. Figure 12, surface view, and Figure 13, optical section, indicate this differential. The prototroch, although present, showed no marked difference in rate of indophenol reaction from adjoining cells (Fig. 12) and the macromeres showed no color at all in the stage of the reaction indicated in the figures.

In very high dilutions of the reagents the embryos were still moving, though slowly, after 16–24 hours and the ectoderm showed no

color but the macromeres were deeply blue. As regards conditions determining this result two possibilities suggest themselves. Either the formation of indophenol is so slow that the ectoderm cells are



FIGS. 12–18.—Indophenol reaction. Figure 12, surface view, Figure 13, optical section of stage beginning movement in egg membrane; gradient apicobasal. Figure 14, early stage of elongation preceding hatching, in surface view; gradient apicobasal; trochal cells do not yet show more rapid reaction than adjoining cells. Figure 15, optical section of larva; apical region, trochal cells and posteroventral lobes show more rapid indophenol reaction than other parts. Figure 16, optical section of somewhat older larva; reaction somewhat more rapid in trochal cells as compared with apical region. Figures 17, 18, optical sections of older larvae; reaction relatively much more rapid in trochal cells than in apical region.

able to dispose of it as rapidly as it forms, or the toxic action of the α naphthol has inhibited or injured the catalyzer of the reaction in the ectoderm cells but not in the less susceptible macromeres. Since

the ectoderm cells were still intact and ciliary movement continued after 16–24 hours the first alternative seems the more probable but further investigation on this point is necessary.

In later stages as elongation begins the indophenol gradient remains the same, that is, decreasing basipetally in the ectoderm (Fig. 14). As larval development progresses, however, the trochal cells begin to form indophenol more rapidly and in the earlier larval period the apical region, the cells of the prototroch, and the posteroventral lobes all develop a deep indophenol blue at about the same time and earlier than other parts (Fig. 15). As larval life continues the reaction becomes more rapid in the trochal cells than in the apical region (Fig. 16) and in the oldest larvae this difference becomes greater (Figs. 17, 18). In general the cells of the posteroventral lobes, except when the lobes are developing, show somewhat less rapid reaction than those of the prototroch and in the general ectoderm a slight anteroposterior indophenol gradient gradually becomes evident in most individuals. Early larvae returned to water before the toxic effect of the agents was strongly marked, or even when they remain in very dilute solutions, show some fading of the indophenol color, beginning at the apical end with a change in color to greenish. This results in an anteroposterior gradient in fading of color. The course of cytolysis and death associated with the indophenol reaction or in α naphthol alone is similar to that observed in other agents and is described in the following section.

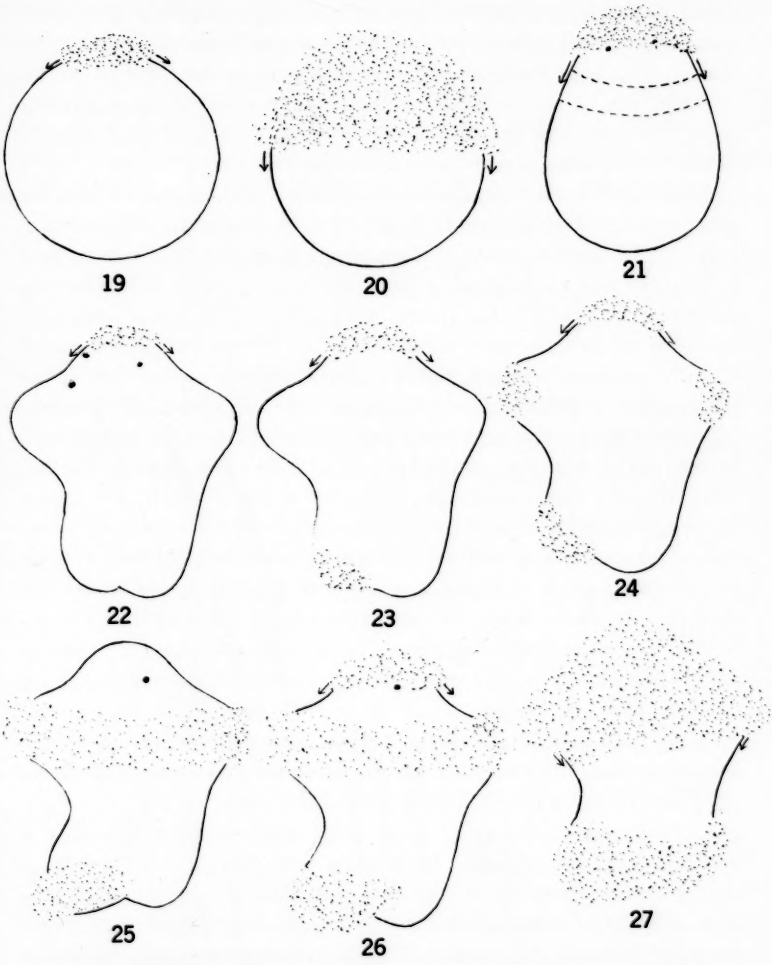
Differential susceptibility in developmental stages.—Differential susceptibility of eggs, embryos, and larvae was determined in seawater solutions of KCN, both alkaline and neutral, in various concentrations from m/1000 to m/100, 10 per cent sea water (tap water killed almost instantaneously), methylene blue, and α naphthol as cytolyzing agents, and the course of cytolysis in connection with the indophenol reaction was also observed. The differential in susceptibility is essentially similar with all these agents and parallels very closely the differential in indophenol formation. It was believed that this group of agents represented a considerable variety in character of toxic action and that differences in susceptibility which were similar or in the same direction would probably indicate non-specific or quantitative physiological differences.

Eggs removed from the uteri to sea water for an hour or two and then placed in KCN m/1000 and m/100 showed a very distinct disintegration gradient beginning at a small area (Fig. 19) and progressing uniformly from this (Fig. 20). When removed from the uteri the eggs vary much in shape, some being elongated, others angular in outline, etc. They stick to glass surfaces and are not easily removed without injury, consequently it is difficult to examine different parts of the surface by rolling them, and no definite indication of the location of the animal pole was observed. As regards the susceptibility gradient, however, it is important to note that disintegration begins in, and progresses from, a very definite area which, so far as could be determined, is not definitely localized in relation to the shape of the egg or to the part in contact with the container surface. Since the susceptibility gradient is independent of these incidental factors it probably represents a real physiological differential in the egg or at least in its peripheral cytoplasm. Eggs which have been fixed after sticking to the bottom usually show injury of that part of the surface which was in contact and disintegration may begin there as soon as, or sooner than, in the physiologically determined region.

In more advanced stages preceding hatching the susceptibility gradient is clearly apicobasal in all the agents used (Fig. 21). At this stage the trochal cells are not distinguished by specially high susceptibility. In the earlier larval stages soon after hatching cytolysis occurs first in the apical region (Fig. 22), and next (in most cases) in the region of the posteroventral lobes which are developing at this stage (Fig. 23) and very slightly later (in some cases at the same time) in some of the cells of the prototroch (Fig. 24).

In the prototroch cytolysis does not always begin with the same cells. The first trochal cells to show swelling and cytolysis are often dorsal but may be ventral or cytolysis may occur in various cells at about the same time (Fig. 24).

Figures 25–27 show stages of cytolysis in the older larvae. In these stages cytolysis usually begins in the trochal cells, both anterior and posterior, somewhat earlier than in the apical region but in some individuals the susceptibility is approximately the same in all three regions (Fig. 26). Except for the high susceptibility of the trochal cells, cytolysis progresses from the apical region basipetally over



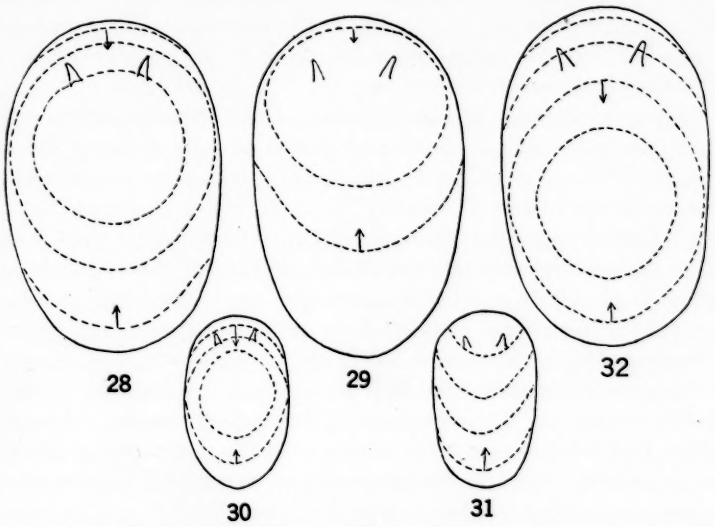
FIGS. 19–27.—Differential susceptibility of embryonic and larval stages: arrows indicate direction of progress of disintegration. Figures 19, 20, stages of cytolysis in eggs from uterus. Figure 21, elongated stage preceding hatching; gradient basipetal; trochal cells not distinguished by specially high susceptibility. Figures 22–24, larva soon after hatching; cytolysis occurs first in apical region and in developing posteroventral lobes, slightly later or about same time in protoproch. Figures 25–27, older larvae: Figure 25, trochal cells most susceptible; Figures 26, 27, later stages of disintegration.

most of the body; but in some larvae the posterior region aside from the trochal cells showed a higher susceptibility than in earlier stages (Fig. 27). Since stages of metamorphosis were not obtained, it could not be determined whether this increase in susceptibility represented the first appearance of the posterior arm of the U-shaped gradient of the adult.

Thus far differences in the course of death and cytolysis which are specific for particular agents have not been observed. Slight differences in the relative susceptibility of apical and trochal cells appear in younger and older larval stages, the apical region normally preceding in the younger, the trochal cells in the older, larvae. Also the course of cytolysis in the trochal cells is not always exactly the same. Usually cytolysis begins in the prototroch slightly earlier than in the posteroventral lobes but in some cases, when the lobes are developing, as in Figure 23, it may occur earlier there than in the prototroch. In the prototroch, also, slight individual differences appear, that is, cytolysis may begin ventrally, dorsally, or elsewhere in the prototroch—perhaps in general most frequently in the ventral cells—but the differences in susceptibility in the different trochal cells are not great. Comparison of Figures 19–27 with Figures 12–18 shows the close parallelism between differential susceptibility and the differential in the indophenol reaction. The indophenol reaction ends in cytolysis and death, the most deeply colored regions undergoing cytolysis first.

Differential susceptibility in mature animals.—Stages of metamorphosis were not observed, the smallest mature animals obtained being 8–10 mm. in length and usually in an early stage of reproductive development, though in a few individuals reproductive organs were not externally visible. Differential susceptibility to the following agents was determined: KCN m/1000 and m/100 approximately, in sea water—alkaline and neutral; chloretone $\frac{1}{4}$ and $\frac{1}{2}$ saturated sea-water solution, tap water, distilled water, hypertonic sea water (salt concentration doubled). Some animals tested had been without food for 20 days, others were freshly collected. In 22 of 25 animals tested in the different agents cytolysis began at the posterior end and progressed anteriorly, and usually a second region of disintegration appeared at the anterior end and progressed posteriorly

(Figs. 28–30). As the figures indicate, the stage of posterior cytolysis at which anterior cytolysis begins differs in different individuals, but apparently without relation to particular agents. Figure 28 represents approximately the usual relation; in Figure 29 anterior cytolysis begins later, in Figure 30 earlier, in relation to posterior cytolysis. In two individuals the disintegration progressed anteriorly over the



FIGS. 28–32.—Differential susceptibility in mature animals. Figures 28–30, usual U-shaped double gradient, higher susceptibility posterior. Figure 31, exceptional case of single gradient from posterior to anterior. Figure 32, exceptional case of higher susceptibility anterior.

whole or almost the whole length of the body, and anterior disintegration was absent or very slight (Fig. 31). In 3 animals (2 small, 1 large), disintegration occurred first at the anterior end, but in 2 of these posterior cytolysis progressed more rapidly, so that the last region to cytolyze was anterior to the middle. In the third, the large animal (Fig. 32), cytolysis progressed posteriorly somewhat more rapidly than anteriorly. Of the 25 animals, 23, or probably 24, that is, 92 or 96 per cent, showed a double, U-shaped susceptibility gradient and in 88 per cent the region of highest susceptibility was pos-

terior. In 96 per cent the least susceptible region was anterior to the middle. It should be noted that in *Stylochus* cytolysis involves all the tissues of the body at much the same time. The whole body softens and "melts" into a semifluid mass.

DISCUSSION

Recent criticisms of methods and results of work in the gradient field make it necessary to consider at this time not only the data on *Stylochus* but also the question of methods, data presented, and conclusions drawn by the critics.

The respiratory gradient of Stylochus.—CO_2 production was determined in 38 series, each series consisting of 3 or 4 lots of pieces (A, B, $C+D$; A, B, C, D; A', B', C', D') representing the different regions of the body. All but 7 of these showed a U-shaped curve, 2 gradients in opposite directions of CO_2 production, at least from A to C. As already suggested, the high rate of the C pieces in Stage II (Table V and Fig. 6) probably results from the developmental and functional activity of the terminal reproductive organ-complex. The data seem to show beyond question that a double respiratory gradient is characteristic of the adult of *Stylochus ijimai*. In the second part of the paper it is shown that a double susceptibility gradient, corresponding closely with the respiratory gradient is characteristic of adult animals similar to stages I–III and that the occasional variations observed in the susceptibility gradient are much like those of the respiratory gradient.

As regards the question of the conditions determining the double gradient, it may be pointed out that in anterior and posterior regions the gut branches and parenchyma in which the ovaries and testes develop constitute a much larger proportion of the total volume than in the pharyngeal region. Apparently the respiratory metabolism of the pharynx when not functioning in the ingestion of food is not high.

The regions posterior to the pharynx, the D pieces or the C and D pieces, have the highest CO_2 production more frequently than the anterior pieces. This difference is probably due in part to the developmental and functional activity of the terminal reproductive organ-complex, as already noted, and in part to the presence in the post-pharyngeal region of a greater number of large, more or less longi-

tudinal gut branches than in the anterior and middle regions, and differential growth of posterior and other regions may also play a part. Perhaps the form of gradient might be altered by several months of starvation and complete disappearance of reproductive organs, but there are no data on this point.

There can be little doubt that the respiratory gradient in the adult *Stylochus* is associated with functional activity and growth in the various regions of the body. Since these may vary considerably in different individuals, even those of approximately the same stage, more individual variation in the gradient is to be expected in adult animals than in early development. Moreover, the form of the gradient in the adult may be very different from that in the embryo.

The indophenol and susceptibility gradients.—Special attention must be called to a few important points. First, the changes in susceptibility and in rate of indophenol formation during development to the larval stage parallel each other very closely; the regions of high susceptibility and rapid indophenol formation apparently correspond very closely to the regions of high physiological activity. During the early stages of development up to the earliest motile stages, the agents used show a single gradient and the apical or anterior region shows the highest susceptibility and the most rapid indophenol formation. As the trochal cells become active they develop a high susceptibility and high rate of indophenol formation, even higher than the apical region in the more advanced larval stages. Even if the differences in susceptibility and rate of indophenol reaction are directly dependent on differences in permeability of the different cells and regions concerned, it can scarcely be doubted that such differences in permeability, if present, must be associated with differences in metabolic activity. But the parallelism of differences in susceptibility to KCN, methylene blue, chloretone, and hypotonic sea water, and between these and the rate of the indophenol reaction in the stages of larval development is evidently not simply a matter of penetration of the various agents. Probably differences in colloidal condition are important factors in differential susceptibility and it is generally believed that colloidal condition and physiological activity of protoplasms are the foundations of structure and function and so indissociable.

Second, at some time during metamorphosis the polar physiological gradient apparently undergoes a partial reversal resulting in the U-shaped gradient of the adult with the highest susceptibility usually at the posterior end and the lowest somewhat variable in position but with rare exceptions anterior to the middle. This change in differential susceptibility from larva to adult is undoubtedly associated with regional physiological differences characteristic of the two stages. The very considerable cellular activity and growth involved in development of the terminal sex organs and the larger proportion of the body volume occupied by branches of the gut posterior to the level of the pharynx are probably important factors in the change. The susceptibility of the pharynx when not functionally active is apparently rather low. The adult individuals of a California species of *Leptoplana* show a somewhat similar susceptibility gradient with high end posterior. The indophenol gradient of that form is not known.

Third, the close parallelism between the susceptibility gradient and the CO_2 production gradients is of interest, as additional evidence that the differences in susceptibility which are not specific for particular agents indicate real and essential differences in physiological condition. In these experiments only those concentrations were used which were lethal within a few hours and in those the regions of highest respiratory rate as indicated by CO_2 production in the adult animals are the most susceptible.

The question of methods.—In this connection it may be noted first that *Stylochus* is much more favorable material than planarian species or various other motile forms for the demonstration of a respiratory gradient. This species lives on tidal flats and may be out of water for considerable periods in nature. It is sluggish in movement and often remains motionless or almost motionless for hours, perhaps for longer periods. In the laboratory it often creeps up the sides of the container above the water level, and in a saturated atmosphere may remain quiet for long periods. When cut into pieces active movement occurs during a few moments, but the pieces, even the anterior piece containing the cephalic ganglia, soon become quiet. This anterior piece is more readily stimulated to movement than the other pieces, which show little capacity for co-ordinated locomotion.

The slow shaking, or rather tilting, in the respirometer apparently has little or no effect in inducing movement with the procedure employed. Moreover, the headless pieces, although they do not regenerate heads, remain alive for days or weeks. Because of these facts it is less difficult to obtain consistent and apparently trustworthy data with this form than with any other motile animal thus far used in work on the respiratory gradient. Parker's colorimetric method for CO_2 determination was used with *Stylochus* partly in order to determine whether with this method and with the necessary precautions a respiratory gradient would be found.

Shearer (1924, 1930), Parker (1929) and Needham (1931) have criticized methods employed and results obtained on respiratory gradients and have presented data or reached conclusions different from those of Hyman and Child. As regards methods, the colorimetric estimation of CO_2 production is first considered. Child (1919*a*, *b*), Robbins and Child (1920), and Child and Hyman (1926) used a colorimetric method for comparative estimation of CO_2 production in *Euplanaria* and *Corymorpha* which involved placing the animals or pieces in the indicator solution. Parker (1929, p. 420) says: "no one conversant with the indicator method would ever employ it in such a way for measuring carbon dioxide since other acids than the one concerned are likely to be involved." Needham (1931, p. 586) says concerning the same method: "But no precautions were used to ensure that the acidity measured was due to carbon dioxide and not to other acids." The criticism that other acids than carbon dioxide may be concerned in the color change of the indicator is easily answered, at least so far as non-gaseous or non-volatile acids are concerned, by bubbling air through the indicator solution or allowing it to stand exposed to air in a thin layer after change in color by, and removal of, the animals or pieces. In one of the earlier papers concerned with this method attention was called to this point and it was stated that with this procedure the color does return (Child, 1919*a*, p. 232). With this precaution the method is adequate for the qualitative and comparative purposes for which it was used. As regards the presence of any other volatile acids than CO_2, Parker's method is open to exactly the same objection as the method of placing the material in the indicator solution. Any volatile or gaseous acid pro-

duced will be present in the air of the respiratory chamber, as well as in the indicator solution. Unfortunately it was not considered necessary to restate in each paper on CO_2 production that the precaution of bringing about the return to the original color was used with the method, and Parker and Needham, without ascertaining the facts, have incorrectly assumed that this check was not applied. It may also be pointed out that as regards production of ammonia, Parker's method is no better than the method of placing the animals in the solution. If ammonia is produced it will be present in the air of the respiratory chamber as well as in the fluid surrounding the animals.

Second, as regards the Winkler method which Hyman has used extensively for determination of oxygen consumption in planarians and other forms, Shearer (1924) says that there are serious objections to it but does not state what they are. Parker (1929, p. 422) agrees with Shearer, but does not state what the objections to this method are. In his later paper Shearer (1930, p. 264) speaks of the error introduced by the discharge of slime and tissue fluids into the water, which he says "is always very great where a large number of animals is employed," but no evidence is presented to show that he has attempted to determine how great this error is or that either he or Parker has ever used this method. Needham (1931, p. 586) questions the value of Hyman's data obtained with the Winkler method on the basis of a personal statement of opinion from Shearer, but again without presenting any evidence in support of his opinion. Hyman considered this point some sixteen years ago in her early work on the Winkler method. The objection is that iodine may be absorbed by organic or inorganic substances in the water and so decrease the oxygen value obtained. Hyman has been fully aware of this point from the beginning of her work with this method and has taken care to determine, by titration for loss of iodine, that this error was negligible in the material with which she worked (Hyman 1916*b*, p. 241; 1932, p. 576). This was done some eight years before Shearer first objected to the Winkler method. In opposition to her actual data there are merely the general opinions of the critics without evidence to support them. It may be pointed out further as regards these criticisms that individuals or pieces of the planarian species with which most of the Winkler work has been done do not

secrete large quantities of slime when undisturbed as they are in the Winkler container; that the slime produced does not immediately dissolve or spread through the water but adheres to the glass and therefore affects the samples taken but little or not at all; third that in the case of the sponge (Hyman, 1925), of *Tubularia* (Hyman, 1926) and *Corymorpha* (Child and Hyman, 1926) there is no appreciable slime production. In the face of the facts it appears that the objections made to the Winkler method are not justified and no other objections have been advanced.

As a matter of fact in cases in which very small amounts of material are not required the Winkler method has various advantages over the ordinary respirometer methods, particularly with material easily excited to movement. The animals are undisturbed in the natural environment and the use of a considerable number of animals or pieces minimizes individual variations. For animals like planarians which are easily stimulated to movement, the respirometer methods involve considerable difficulties. The shaking, particularly the starting and stopping, in connection with readings tends to induce movement and even in case it does not induce movement its effect on respiration is not known. Some of these difficulties are evident in the work of Parker and Shearer (see also Duryee, 1932). Parker (1929, p. 417) says concerning the planarians which he used: "ordinarily they remained quietly in the bottom of the scoop." And again concerning *Nereis* pieces (p. 413) that they remained in "relative quiescence." "Ordinarily" and "relative quiescence" are not usually considered an adequate basis for regarding data as quantitatively accurate. Shearer (1930) was unable to eliminate movement in his respirometer work on flatworms and although his data indicate a gradient he concludes that they are invalidated by movement. On page 263 he says that the ratio of oxygen consumption in pieces from different body levels was "just about the same as the relative amount of movement of these parts in the course of measurement." One wonders how the amount of movement was measured. Moreover, the decrease in the amount of movement from anterior to posterior pieces requires explanation and suggests a gradient expression of some kind.

Any method which involves cutting animals into pieces and then

trying to determine what the rate of respiration was in the different regions of the body before cutting must be regarded as of doubtful value, and even reasonably or probably trustworthy results can be obtained with such methods only by use of all possible precautions. Unfortunately, the respirometer methods as ordinarily employed though theoretically accurate, are not well adapted to this sort of work with actively motile material. Shearer does not state whether he removed the heads of the flatworm used. If he did not, his failure to do so and the use of relatively long pieces account in part for his difficulties as regards movement. With animals as easily excited to movement as planarians we have always found it advisable to remove the heads and to make the respiratory determinations or estimations on postcephalic pieces. The difference in motor activity following section in pieces from different body levels has been known for years to Hyman, Child, and others who have worked with planarians at Chicago, and has been regarded as one expression of a gradient of some sort. But it decreases with decrease in length of piece and with length of time after section, and repeated determinations on the same material have shown that with proper care it becomes negligible as a complicating factor. A former student at Chicago, Dr. Fraps, has found it advisable to use pieces of planarians only about 2 mm. in length for respirometer work. In these pieces motor activity is practically absent within an hour or two after section and repeated determinations on the same material vary only about 3 per cent. These short pieces often show no visible movement for several days after the stimulation of section is over. It does not follow, of course, that isolated pieces of other species behave in the same way, but Shearer's attempts to eliminate movement are not sufficient to throw any light on that point.

Moreover, Shearer gives only averages for the planarian, and apparently data for only one individual of *Thysanozoon*. It is impossible to determine from these data how great the scatter of individual determinations was and whether the data as they stand are significant. Apparently he did not attempt to determine whether section of the animals increased oxygen consumption. He gives no information concerning the time between section and determinations and there is no evidence that he made successive determinations on the

same material at different periods after section. Apparently he did not even try pieces differing considerably in length to see if movement could be eliminated. We cannot agree with Needham (1931, p. 589) as to the accuracy of Shearer's work, regardless of the accuracy of the method.

As regards differential susceptibility, the differentials in $KMnO_4$ reduction, the indophenol reaction, and the methylene blue differential, it may be noted that none of these differentials are regarded as demonstrating completely the existence of a metabolic gradient. The later development of work in this field has shown beyond any question that there is a remarkable parallelism in the differential susceptibility to certain ranges of concentration or degree of action of a large number of both physical and chemical agents, and also between susceptibility and physiological condition as indicated not only by development but by functional activity. Moreover, there is a close parallelism between the susceptibility gradients and the indophenol, permanganate, and methylene blue gradients. It has been demonstrated that differences in penetration do not account for the results. All of these methods show the existence of gradients of some sort, as Needham admits, and if all the facts are taken into consideration it is difficult to avoid the conclusion that in general a metabolic gradient parallels more or less closely the various indications of gradients which these method demonstrate.

Shearer (1930, p. 267) finds it "hard to believe" that different agents such as KCN, alcohol, ether, $KMnO_4$, "each with a totally different action on the respiratory system of the living cell, should demonstrate the same gradient in this simple manner." As a matter of fact, it is not at all necessary to assume that all these agents do demonstrate "the same gradient" or that they all act directly on the respiratory system of the cell, and I think that such assumption has not been made. What they do demonstrate is parallel gradients or parallel aspects or functions of a graded condition in the protoplasm, and our experience with these and other agents has led us to believe that a metabolic gradient is one aspect, expression, or function of this graded condition. Concerning the facts of differential susceptibility there can be no question, as one who is willing to test the matter for himself with a wide range of concentrations of different

agents and an open mind can readily ascertain. Parker's and Shearer's criticisms of the susceptibility method to the effect that the differences in susceptibility indicate merely differences in permeability, or that in the case of *Nereis* (Parker, 1929) the agent enters through the mouth and anus, do not account for the following facts. First differential susceptibility to ultra-violet, X-rays, radium, temperature extremes, lack of oxygen, except as these agents may alter permeability, does not depend upon differential permeability. Second, it has been shown that agents to which the normal cell surface is highly permeable and those which penetrate only by altering the surface give parallel gradients. Third, low concentrations of certain agents have been shown to give gradients in opposite directions from a higher range of concentrations. Fourth, in relation to the case of *Nereis*, it may be pointed out that even if the agent enters through mouth and anus this is not true in many other cases, e.g., planarians and other flatworms, many embryonic stages, etc. Usually the mouth, at least, is tightly closed in the presence of the lethal agent. Fifth, the experiments on the modification of development through differential susceptibility certainly indicate, in many cases, the presence of gradients which involve metabolism of some kind.

The nitroprusside reaction was used with *Corymorpha* in a purely qualitative manner as various other workers have used it in the attempt to determine whether any evidence of glutathione or SH gradient could be observed (Child and Hyman, 1926). When the data were published it was noted that the reaction was probably not specific for glutathione. Needham's criticism (1931, p. 587) that the results cannot be accepted "in the absence of exact quantitative data especially considering the unspecific character of the nitroprusside test applied to an intact animal" appears rather captious. Surely it is permissible and of some interest to note that a gradient was observed, even if quantitative methods were not employed. Moreover, the presence of a gradient is the chief point of the experiment: whether it represents glutathione or some other SH compound or something else is of minor importance in this case.

As regards the indophenol gradient which has been demonstrated in many cases and the gradient in reduction of methylene blue, Needham (1931, p. 589) says: "these observations cannot by any

means permit of conclusions about gradients of metabolic rate," but gives no evidence in support of his statement. In view of the fact that the gradients demonstrated by these methods parallel the susceptibility and permanganate gradients this statement seems somewhat extreme. The indophenol oxidase is regarded by various authors as of considerable importance in oxidation. Shearer, for example (1930, p. 267) says that "it accounts for two-thirds of the total respiration of the cell" and other references could be given. In the light of these facts Needham's summary dismissal of the evidence obtained by these methods seems to call for some basis of evidence. The great need in this field, however, is for a method of direct determination of metabolic or respiratory rate in different regions of an embryo, an egg, or other cell, without cutting it into pieces or disturbing it otherwise. In the absence of such a method, the methods available have been used and the results obtained on early developmental stages when taken altogether are at least highly suggestive or indicative, though not directly demonstrative, of the existence of a gradient in which metabolism is an essential factor. If Needham's assertion that these methods do not permit conclusions concerning a metabolic gradient means that they do not constitute a complete demonstration, one may agree with him. But if consistency and agreement of many different lines of evidence have any value it becomes difficult to escape the conclusion that metabolism is an essential factor in the gradients which these and other methods demonstrate.

Gradients in Adult Animals.—The earliest work by Child on the physiological gradients was done on adult animals, but the attempt was soon made to determine whether gradients are present in the early stages of development. It was found that physiological gradients of some kind which show the same characteristics as those of adult individuals are present in developmental stages of axiate organisms, and that as new organs and parts appear, and differentiation progresses, the original gradients may be altered or obliterated and new gradients may appear in other directions. In any organism with specialized organs in different regions the respiratory metabolism of any region is the sum of the respiratory metabolisms of the different organs present in that region. Whether a respiratory gradient is or is not present along the polar axis of the body will depend

on the activities of the various organs and the directions of the respiratory gradients in the different organs of that regions, if such are present. The presence or absence of a longitudinal respiratory gradient in the adult of one species does not permit the conclusion that a similar gradient must be present or absent in other species, even in those closely related to the first. Slight differences in relative volume or activity of particular organs may determine the presence or absence of a general gradient. For example, a gradient of some sort is present in the body wall of *Euplanaria*, as indicated by differences in lethal susceptibility and other differences which show gradation from the head posteriorly. In the gut, however, similar gradient expressions decrease from the middle region both anteriorly and posteriorly. If respiration is a factor in these gradients it is not difficult to see that in anterior and middle regions the two gradients may balance each other, and no gradient at all may be found in some species, while in others a general gradient may appear in one direction or the other. It is only in relatively simple organisms or regions like the elongated simple sponge or the hydroid stem that we can hope to avoid such complications.

It is evident, therefore, that from the present standpoint of the gradient theory the presence or absence of a longitudinal respiratory or other gradient in adult individuals of organisms with different organs at different body levels is of no fundamental importance, but merely a point to be determined for each species, and of significance chiefly in relation to the gradients of earlier developmental stages as showing whether or what changes have occurred in the course of development. The presence of an anteroposterior respiratory or other gradient in the adult of one species and its absence in another, granting that the demonstration is complete in both cases and free from complicating factors, is significant from the gradient standpoint only as a specific difference. Since this is the case it is evident that Parker's data and Shearer's conclusions concerning movement, even if entirely correct, do not constitute evidence against the general gradient theory, for in all cases their data were obtained with different species from our own, with the exception of *Nereis*, where there is disagreement between Parker's and Hyman's data (Parker, 1929; Hyman, 1932) which will be considered below.

The point of chief importance in the gradient concept is the relation of development along a physiological axis to a gradient, and the evidence indicates that the gradient is a fundamental and determining factor in axiate development. The condition in the adult is the resultant of all the developmental and functional processes and the greater the structural and functional complication, the less likely is the body as a whole to show the simple gradient of early developmental stages. It seems probable that if the primary gradient persists anywhere in the adult body it is likely to be in the central nervous system.

Needham, on page 584, regards the change in views concerning the relation between oxidation and growth, and the fact that growth-rate and metabolic rate may take a diametrically opposite course as evidence against the metabolic character of the axial gradient. His argument fails to take account of the fact that the gradients with which we have been concerned are not necessarily or always growth gradients, since they may be present before growth begins, and often in the later embryonic and in postembryonic stages they may be opposite in direction to the growth gradients. For example, it is evident to anyone who compares individuals of planarians of different size that the rate of growth in length increases in general posteriorly. (See also Abeloos, 1928.) The susceptibility and other axial gradient expressions, however, are wholly or in part opposite in direction to this growth gradient. The same is true for various other cases. In the earlier embryonic stages, however, the various gradient expressions correspond more closely in direction but growth is by no means a necessary factor in their existence, and they may be present before embryonic growth begins. Needham's criticism is wholly misdirected as regards this point.

Data and conclusions of recent critics.—As regards Shearer (1924, 1930) it is of interest to note that both his earlier data on earthworm and chick and the later data on flatworms show a gradient, but he concludes that these data are not trustworthy. In his earlier paper he regarded the drop in oxygen consumption which occurred in the pieces of chick embryos after some thirty minutes in the respirometer as due to disintegration of the pieces (p. 149). In his later paper (p. 261) he concludes that the higher rate of respiration of an-

terior pieces of the chick embryo was due to their more rapid cytolysis and states that cytolyzing tissue always consumes a large amount of oxygen. Hyman (1932) has called attention to some other apparent inconsistencies in Shearer's paper and has shown that the respiratory differences in certain data of her own are statistically significant, though he had criticized them as being too small to be considered as significant.

On page 266 Shearer (1930), after citing the work of various authors on oxygen consumption and heat production during development, reaches the following conclusion:

> It is evident from facts of this character that little or no energy is involved in the morphological organization of the embryo, all being probably expended, as Warburg long ago suggested, in keeping the constituents of the protoplasm in being as a physical system. During the course of development it is the quantity of living material manufactured, not its quality, that uses the energy liberated by the oxygen consumption. Morphological organization has nothing to do with metabolism.

This statement of Shearer's concerns exactly the point on which much of the misunderstanding concerning the gradient conception has arisen. The evidence concerning the existence of gradients and the part played by them in development points very definitely to the conclusion that "keeping the constituents of the protoplasm in being as a physical system" involves maintenance of the gradient or gradients present in that protoplasm. When the gradients are altered the course and character of development are altered, and when the gradients are obliterated axiate development does not occur. When new gradients are determined new physiological axes are determined and differential growth and morphogenesis follow in definite relation to the new gradient. The evidence indicates that this differential growth and morphogenesis are determined by the new gradient. The maintenance of the protoplasmic system with its gradient or gradients probably requires oxidative metabolism and the expenditure of energy, as Shearer has stated. If this is true then the gradient is fundamentally and necessarily a metabolic gradient. Since such a metabolic gradient is inevitably associated with the protoplasmic substratum, and in maintaining the protoplasmic system determines various gradient expressions in this substratum, perhaps gradients in colloidal dispersion, viscosity, water content, electrolyte

content, etc., the gradient may be altered or obliterated or parallel gradient effects may be made evident as death gradients, tolerance gradients, gradients in inhibition of development, etc., by the action of many physical and chemical agents which act on the protoplasmic system in very different ways, even though they do not directly affect the respiratory system at all. Apparently neither Shearer nor Needham has understood this essential point in the gradient conception, although, as we believe, it is amply supported by many different lines of evidence, all of which agree. The facts of developmental modification in relation to differential susceptibility to many different chemical and physical agents, the obliteration of gradients, the determination of new gradients by various external factors, the death gradients, and gradients in threshold of tolerance, all constitute evidence for this conception of physiological gradients.

Moreover, when Shearer says that "it is the quantity of living material manufactured, not its quality, that uses the energy liberated by oxygen consumption" he apparently fails to realize that these words imply the existence of a metabolic gradient, at least in the earlier embryonic stages, for it is certainly true that during these stages the quantity of living material manufactured is in general greater, often very much greater, in the apical or anterior region of the embryo than elsewhere. If Shearer's statement is correct the conclusion that an apicobasal or anteroposterior metabolic gradient exists generally in the earlier embryonic stages apparently cannot be avoided. In fact, Shearer's argument seems to lead directly to such a conclusion and we are indebted to him for putting the case so clearly.

The evidence concerning the relation of the gradient to development also indicates that maintenance of the gradient is the primary and fundamental factor in determining differential growth and morphogenesis. If the gradient is maintained differential growth and morphogenesis necessarily follow. Whether or not growth or morphogenesis as distinguished from maintenance of the gradient involves expenditure of energy is for the gradient conception a matter of secondary importance, and any conclusion based on evidence is acceptable. However, Shearer's assertion that morphological organization has nothing to do with metabolism seems rather extreme.

Even if metabolism is not immediately and directly concerned it is certainly indirectly a fundamental factor in morphological organization. Parker evidently means much the same thing as Shearer when he says that "the metabolic activity of the organism is not a true formative process but the result of such a process" (Parker, 1929, p. 424). Even if it be true that little or no energy expenditure is involved in morphogenesis, the form of statement in both cases seems at least somewhat unfortunate. Certainly chemical reactions are essential factors in the formation of particular organs which differ in constitution from embryonic protoplasm, but it may well be that these reactions involve little expenditure of energy.

In connection with Shearer's argument concerning the quantity of living material manufactured in relation to the energy liberated by oxygen consumption it is of interest to observe that according to Needham (p. 584) the idea that oxidative processes and growth are closely allied has been abandoned by all physiologists because it has been shown to be incorrect. Shearer seems to imply that oxidation is an essential factor in growth, and we believe that somewhat similar views have been recently advanced by others.

Parker (1929) presents data on CO_2 production in pieces of *Planaria maculata* (*Euplanaria novangliae*), *Nereis virens*, *Sagartia luciae* and *Metridium marginatum*. No definite gradient is found in *Euplanaria*, *Sagartia*, and *Metridium*, and in *Nereis* the CO_2 production of anterior and posterior pieces is lower than that of pieces from the middle region; and the susceptibility of the two ends is higher than that of the middle. Hyman and Galigher (1921) found that the oxygen consumption of the two ends of *Nereis* is higher than that of the middle. Hyman (1932) has already discussed the data on *Nereis* and has obtained new data which agree with the earlier data of Hyman and Galigher as regards the presence and character of gradient. Only one point concerning *Nereis* need be considered here. According to the graph on page 415 of Parker's paper the CO_2 production of whole worms is almost as high as that of the middle pieces alone, while the CO_2 production of the end pieces is much lower. The graph indicates that a marked decrease in CO_2 production has occurred since section, almost entirely in the end pieces. Parker found the ends of the body more susceptible than the middle to KCN.

The graph suggests that they are also more susceptible to the loss of blood and other effects of section. They were kept "a few hours" between section and CO_2 determination "to recover from the operation." Apparently they did not recover, particularly the anterior and posterior pieces, and the gradient which Parker has found is probably the reverse of the normal gradient. In the laboratory at Chicago we have brought about reversal of the susceptibility gradient in fresh water oligochete annelids merely by keeping them in clear standing water with no mud and no food for a few days. In the newly collected animals the two ends were most susceptible but after a few days under the conditions described the susceptibility was lower and the middle region was most susceptible. Parker does not give the size of his animals but says they were small. Hyman used animals 3–10 inches in length in her different determinations. If Parker's animals were smaller than this they were doubtless more susceptible. In any case it can scarcely be doubted that respiration is likely to be more nearly normal when the pieces remain undisturbed in water, as in Hyman's determinations, than in the scoop of Parker's respirometer exposed to saturated atmosphere and continuously shaken.

Parker's data for *Euplanaria novangliae* may be entirely correct for the species. At Chicago we have found the gradient less distinct in this species than in *Euplanaria dorotocephala*, but it shows a very distinct gradient in reconstitution at different body levels. There is, however, one point which makes the value of Parker's data for any purpose quite uncertain. According to a personal communication, he did not determine whether the animals were asexual or in some stage of sexual development. This species differs from *E. dorotocephala* in that it develops reproductive organs in summer, at least in many localities, while in the region about Chicago *E. dorotocephala* has rarely been found with reproductive organs during some twenty-five years. The terminal reproductive organ complex develops in the region of the body which in asexual individuals of *E. dorotocephala* shows the lowest respiratory metabolism (Hyman, 1923) and it is certainly not to be expected that the development, growth, and functional activity of this complex will have no effect on the respiratory metabolism of this region. It was shown above that in *Stylochus* the CO_2 production of the region in which the terminal organ complex

is situated shows a great increase in CO_2 production relative to other body levels during the period of greatest activity of these organs. In view of the fact that Parker gives no information concerning the presence, absence, or stage of development of reproductive organs in his material, his data on CO_2 production, assuming that they are correct, show only that if gradients are present in different organs or tissues they more or less compensate each other as regard total CO_2 production. The data do not even constitute conclusive evidence that gradients are characteristically absent in the asexual adult of this species.

The absence, according to Parker's data, of any definite differences in CO_2 production in distal and proximal halves of the actinians, *Sagartia* and *Metridium*, is also without any definite bearing on the question of presence or absence of a gradient in these animals. The determination of CO_2 production in only 2 pieces (a distal and a proximal half), more or less, does not show whether any gradient is present or not. At least 3 pieces would be necessary to determine this point. If the CO_2 determinations in the case of *Stylochus* had been made on only 2 pieces, anterior and posterior halves, certainly no definite gradient would have been observed. Because of its secretory activity or other functional characteristics which arise in the course of development, the foot region of the actinian may become secondarily a region of high respiratory activity, and the intermediate region may have a lower rate, that is, there may be a U-shaped gradient in the adult actinian. But whether or not this is true cannot be determined by separation of the body into 2 pieces only, as Parker has done. At least 3 pieces must be used. Moreover, there is nothing in the account of experiments on actinians to show that the high CO_2 production of the proximal half was not due to laceration or other injury of the foot region, such as commonly occurs in detaching the animals. There is also the possibility that the activity of the foot region may be increased by detachment, even without injury. This seems to be the case in *Hydra*. None of these various possibilities are investigated experimentally or even mentioned by Parker, nor is there any evidence from his paper that he attempted to determine whether the pieces were stimulated by section. We might expect that the oral piece would be little affected by section and the

aboral piece more or less stimulated. As they stand these data on actinians mean nothing.

Parker's arguments from the data and in general are largely invalidated by his fundamental misunderstanding of the gradient theory. Apparently he believes that the theory demands a longitudinal gradient in the adult of every species. As pointed out above, and as repeatedly stated in earlier publications, this is far from the case. Of particular interest in this connection is his argument concerning the nervous system. He holds that the metabolism of the central nervous system in adult animals is low. In support of this view attention is called to the low metabolism of peripheral nerves, but the possibility that the metabolism of the cell body may be higher than that of the fiber is not considered. There is, however, considerable literature on the respiratory metabolism of the central nervous system which does not support Parker's conclusion, but which he does not discuss. Hyman (1932) has called attention to some of it. It is impossible to consider the literature at length here but it may be noted that, according to Gerard (1928, p. 11; 1931, p. 73 and references there given), the oxygen consumption of gray matter is about seventy times that of nerve fibers. In a more recent personal communication Dr. Gerard states that according to the data now available it appears that gray matter consumes from 20 to 70 times as much oxygen as nerve fibers, according to material and conditions of determination. Moreover, it is a well-known fact that the brain receives more oxygen per unit of weight than any other organ except the thyroid and adrenal and perhaps other endocrine glands. These facts, as well as the high susceptibility of the brain to lack of oxygen, do not support Parker's argument, and it is somewhat surprising to find a student of the nervous system apparently ignorant of them. In short it seems to be established beyond question that the nerve cell has a very intense respiratory metabolism, at least in the so-called higher animals. But whatever the facts may be, the question is of no fundamental importance to the gradient theory. This theory has maintained only that the central nervous system arises in those regions which show the highest metabolism in early developmental stages, and whether or not they remain the regions of highest metabolism throughout life is merely a question of fact to be determined

by experiment and of no theoretical significance. Hyman called attention to this point some seventeen years ago (Hyman, 1916*a*) and it has been emphasized many times since. As one point in this argument for low metabolism of the nervous system Parker (1929, p. 424) makes the remarkable statement that "on exposure to cold we warm ourselves by exercising our muscles and not by thinking hard." Considering only the difference in volume of nervous system and muscles this is, to say the least, a rather surprising statement in this connection.

On page 423 Parker also argues that "the high metabolic point in a complex animal must be thought of as shifting in location from moment to moment," and says further that "this diversity must be regarded as the outgrowth of a system in which relative homogeneity is the rule and in which very little of that fixed metabolic diversity implied by Child's gradient hypothesis is present." Actually, however, the gradient hypothesis has never implied a fixed metabolic diversity in adult animals. If there were any such implication we would not regard it as desirable to make certain that digestive activity is decreased to a minimum, and the gut emptied as far as possible before undertaking respiratory determinations on planarians. It has been stated repeatedly that development and functional activity of particular organs must bring about changes from the primary embryonic situation. With all due consideration, it seems permissible to suggest that a little more familiarity with the gradient theory than is evident in Parker's discussion is desirable before attempting criticism.

It seems to be necessary to point out once more: first, that the question whether the primary embryonic gradient or whether any respiratory or other gradient or gradients are present in the adult longitudinal axis can be determined only by experimental investigation of each species with the most complete control possible of experimental and physiological conditions which may affect respiratory rate of the different regions of the body; second, that even with such control data on respiration of isolated pieces from different body levels may be complicated by the presence of gradients in opposite directions in different organs and by the functional activities of particular organs; third, that at best such data constitute

only contributory rather than final evidence on the question of gradients.

In conclusion it is of interest to note to what extent opinion rather than actual evidence is concerned in these criticisms. Shearer's criticism of the Winkler method seems to be based on opinion rather than actual investigation. He discards both his earlier and his later experimental data because of his opinion that they are invalidated by complicating factors, but makes little attempt to determine whether modification of technique would give better results. Parker's whole discussion involves a fundamental misunderstanding of the gradient theory in consequence of which he fails to realize that his experimental data are of no importance in relation to it. Needham's criticisms consist largely of statements without presentation of evidence to support them, except for his quotation of Shearer's opinion regarding the Winkler method.

As the situation stands at present there are data showing the presence of a respiratory gradient in adult individuals of the following forms: *Grantia* (Hyman, 1925), *Tubularia* (Hyman, 1926), *Corymorpha* (Child and Hyman, 1926), *Euplanaria dorotocephala* (Robbins and Child, 1920; Hyman, 1923), *Stylochus ijimai* (present paper), *Nereis virens*, *Nereis vexillosa*, *Lumbriculus inconstans* (Hyman, and Galigher, 1921), confirmatory data on *Nereis virens* (Hyman, 1932), earthworms, species not given (Perkins, 1929),[2] the earthworm *Allolobophora foetida* (Watanabe, 1931), also data on the earthworm *Pheretima hilgendorfi* obtained by Watanabe but not yet published. The data on *Stylochus*, *Allolobophora*, and *Pheretima* were obtained by Parker's method. As regards Perkins' data it should be noted that the oxygen consumption gradient is slight and does not correspond with the gradients of total iodine equivalence, extractable sulfhydryl, and total sulphur. As Perkins suggests, however, the oxygen consumption gradient doubtless includes other oxidation systems than those associated with growth.

Against these positive data we must place: first, Parker's data on *Nereis*, *Euplanaria*, and actinians, the value of which has been sufficiently discussed above; second, Shearer's opinion that his

[2] In a personal communication to one of us Perkins stated that two genera of earthworms, *Lumbricus sp.* and *Allolobophora sp.* were used in his determinations.

earlier positive data are invalid and his later data the result of differences in motor activity; third, the objections raised by Shearer, Parker, and Needham to the Winkler method which indicate that these authors were ignorant of the fact that long before the objections were raised, Hyman was fully aware of the point concerned, and had determined experimentally that the conditions on which the objections were based did not appreciably affect the oxygen values. Since these objections to the Winkler method have been shown to be groundless so far as Hyman's data are concerned and since the data obtained on *Stylochus* and two species of earthworm by Parker's method give highly consistent and positive evidence for a gradient, it does not appear possible to escape the conclusion that a respiratory gradient is present in the adult individuals of some organisms. Whether or not such a gradient is present in any particular species can be determined only by adequately controlled experiment, and even if a gradient of total respiration is not present respiratory gradients in different directions may be present in different organs or organ systems. Some direct evidence of respiratory organ gradients has already been obtained, for example in the central nervous system (MacArthur and Jones, 1917; Bass, 1923) and in the mammalian intestine (Alvarez, 1928).

We regret the necessity for the preceding discussion, but that necessity has arisen because of the superficial and mistaken character of the criticisms with which it is concerned. Criticisms such as these, which show ignorance of, or failure to consider, available facts, which present as evidence meaningless data resulting from incorrect procedure or inadequate analytic experimentation, which make unjustified accusations of carelessness, looseness, and ignorance, and which in many cases rest on personal opinion and assertion without evidence, cannot be permitted to go unchallenged.

SUMMARY

1. The paper includes: (*a*) data on the longitudinal respiratory gradient of the polyclad *Stylochus ijimai* in different stages of development of reproductive organs as indicated by the CO_2 production of pieces from different levels determined by Parker's method; (*b*) on the indophenol and susceptibility gradient of embryonic and larval

stages; (*c*) on the susceptibility gradient of animals in various reproductive stages.

2. CO_2 production is greatly increased by section. During the first hour following section it decreases very rapidly almost to the level of the intact animal. Further decrease is very gradual and slight and approximate constancy is soon attained.

3. Determinations of CO_2 production of pieces at different periods following section show wide variations immediately after section, but a high degree of uniformity in each lot 2–6 hours after section.

4. The characteristic respiratory gradient as indicated by the CO_2 production of pieces from different body levels is a U-shaped double gradient with regions of higher respiration anterior and posterior, the region of highest rate being more frequently posterior than anterior.

5. The form of the gradient differs somewhat in different individuals and in different stages of development of reproductive organs. In Stage II when the developmental and functional activity of the terminal reproductive organ complex is high the piece containing this complex shows in general the highest CO_2 production of the body, much higher than in earlier or later stages.

6. Determinations on intact animals in the three stages of reproductive development showed a decrease in rate of CO_2 production from earlier to later stages, indicating physiological senescence.

7. In embryonic stages preceding hatching the rate of intracellular indophenol reaction and the susceptibility to KCN, methylene blue, 10 per cent sea water, and naphthol decreases from the apical region basipetally. After hatching, as the posteroventral lobes develop and the ciliated cells of the prototroch and paratroch become active, these regions show an increase in rate of indophenol reaction and of susceptibility as compared with the apical region, and in older larvae the rate and susceptibility of the posterior end shows an increase, suggesting the appearance of a double gradient.

8. The susceptibility gradient in mature animals is with rare exceptions a U-shaped gradient similar to the gradient of CO_2 production.

9. A discussion of some recent criticisms of the gradient data and theory is appended.

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THE RELATION BETWEEN THE FORM OF THE LIVING CELL AND THE NUCLEAR PHASES OF DIVISION IN AMOEBA PROTEUS (LEIDY)

(Nineteen figures)

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DESPITE the fact that *Amoeba proteus* (Leidy) is one of the most widely studied of the non-pathogenic Protozoa, the process of division in this form has not been given the attention that one would expect. There is consequently a dearth of reliable accounts of this process and a conflict in the descriptions of the typical mode of vegetative reproduction. Awerinzew (1904) described division in a form which he identified as *proteus* but, as it had an ovoid nucleus, it cannot be this but is possibly *Amoeba dubia* (Sch.). Doflein (1918) gives a clear description of nuclear division but, as we shall point out later, apparently confused resting polynucleate cells with dividing cells, and came to the conclusion, which we believe to be erroneus, that the normal method of vegetative reproduction was by *multiple* fission. Carter (1912) described a single division stage which she designated as a metaphase. From her description of the amoebae in the culture used it seems probable that the form was, as with Awerinzew, *dubia*. In fact she herself identified it as such. Taylor (1923) found no mitotic division, but pictured a series of peculiar nuclear forms that apparently lead to a form of division approaching amitosis. Levy (1924), who made a statistical study of the same species we used, secured probably from the same source, states that while he observed cell division he found no nuclei in his fixed preparations, but merely scattered granules, and suggests that during division a complete dispersal of the nuclear material occurs. Thus it is obvious that there is need for a careful and thorough investigation of vegetative reproduction in *A. proteus*.

In this laboratory investigations as to the effect of certain reagents on division in *Amoeba proteus*, Voegtlin and Chalkley (1930), Chalk-

ley and Voegtlin (1932), and a study of the relation of cell growth to division, Chalkley (1931), have been in progress. During the course of these in the autumn of 1931 one of us (C.) observed several dividing amoebae and by careful observation soon found it possible to select the dividing forms from culture. It occurred to us that if a careful study were made the of process of division it would be possible to clarify the situation outlined above. We also hoped that a thorough knowledge of the facts might provide a basis for the technique of a more detailed attack on the chemical factors involved in cell division. The suggestion met with hearty approval from Professor Carl Voegtlin, to whom thanks are due for his continued interest and encouragement of the work, and we began the investigation, the results of which are presented here.

MATERIAL AND METHODS

The material used was a strain of *Amoeba proteus* descended from a single cell isolated in this laboratory about a year ago, from a mass culture originally obtained from the Biological Laboratory of Johns Hopkins University through the kindness of Professor S. O. Mast. The amoebae were mass cultured in flat-bottomed glass dishes 17–18 cm. in diameter. The culture medium was as follows: NaCl 0.1 gr., KCl 0.004 gr., $CaCl_2$ 0.006 gr., H_2O glass distilled 1,000 cc.; to this was added 2–3 cc. of Clark and Lubs phosphate buffer m/20, pH, 7.0–6.8. The dishes were filled to a depth of approximately 1 cm. and 8–12 grains of polished rice were added. Each culture was then "seeded" with 50 or more amoebae and covered to prevent evaporation. The cultures were kept in diffuse light at room temperature which averaged approximately 24° C. Such cultures are of rapid development reaching their full vigor in a period of from 2 to 3 weeks. They will provide ample material for the study of division only over the relatively short period of a week or so. After this the rate of division slows markedly, probably due to crowding, and the culture must be renewed, or at least radically depleted in population, and saline and rice must be added before the fission rate will again rise sufficiently to provide a good supply of dividing forms.

The dividing amoebae were selected from these cultures by means of a Bausch and Lomb binocular dissecting microscope using re-

flected light and a black background. They are readily distinguishable from other organisms under a magnification of 30 diameters. Observations of the living cell were made *in situ* in the culture dish under the binocular when detail was not needed and it seemed desirable to avoid disturbance of the organism. When detail was needed or measurements were to be made, an organism was carefully transferred to a depression slide by means of a capillary pipette and observed under a compound microscope. The fixing and staining of organisms was done as follows:

Cover glasses, cleaned in dichromate solution and alcohol, were used exclusively; very clean covers are essential. The organism to be stained should be placed at the center of the cover glass in a very small drop of culture fluid. Several of the common fixatives were used but none gave as satisfactory results as Carnoy and Lebrun's fluid. This solution gives excellent fixation and at the same time has the added advantage of making the amoebae adhere firmly to the cover glass. If care is taken to see that the fixative is *dropped from above directly upon the animal* with the cover glass held at an angle so that the culture fluid is rapidly displaced, very few of the organisms will be lost in subsequent operations. A few minutes' (15–20) washing in 70 per cent alcohol served to rid the material of bichloride crystals. The amoebae can be taken directly from the 70 per cent alcohol to the stain.

At the suggestion of Dr. R. D. Lillie of this Institute, ferric chloride haematoxylin stain was tried. The stain giving the best results was slightly modified from the original formula. It was made as follows:

Solution A:

0.33 per cent $FeCl_3 \cdot 6H_2O$	4 cc.
Concentrated HCl	4 cc.
Distilled H_2O	95 cc.

Solution B:

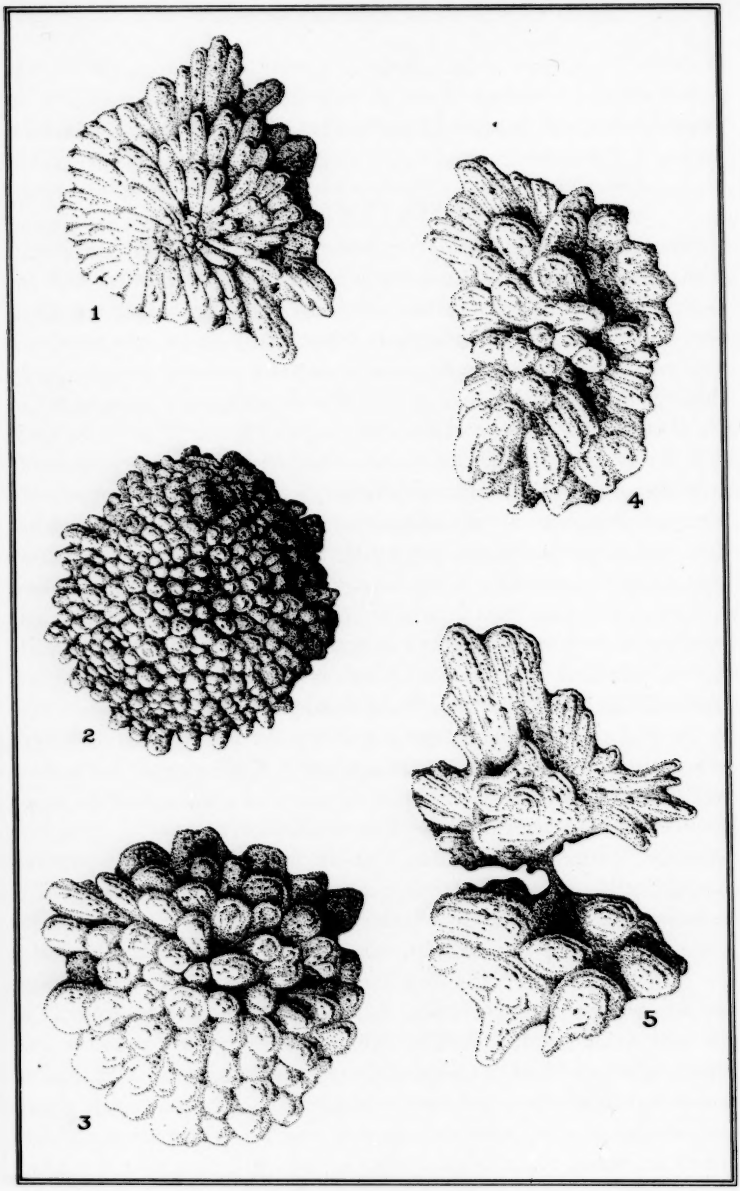
1 per cent Grubler's haematoxylin in 95 per cent alcohol.
The unripened solution is better than old material.

For use mix equal parts of *A* and *B* and use at once. The mixture is good for 24–48 hours, but is best used fresh. Three minutes in the stain proved sufficient for the organism used. Rinse in acidulated water (3 drops HCl in 200 cc. H_2O) then in tap water, dehydrate

and clear in acetone and benzene, and mount in balsam. While not suited for fine cytological detail this stain gives a rapid and reliable method for following the division phases of the cell nucleus.

THE CYTOPLASMIC PHENOMENA OF DIVISION AS SEEN IN THE LIVING CELL

Amoebae near the time of formation of the division sphere present a characteristic appearance. They are sluggish in motion and are beset with very numerous fine pseudopodia. They exhibit a tendency to acquire a peculiar form in which the pseudopodia assume a somewhat stratified order and become arranged more or less radially about a distinctly hyaline spot, which is composed apparently of the contractile vacuole and the nucleus (see Fig. 1). This condition is apparently somewhat slowly attained and therefore, except as indicative of a relatively mature condition, is not of prime importance for the selection of the dividing form from culture. This configuration may be acquired and lost several times over a period of hours before the initial stages of division actually begin. When the division process is finally initiated the organism ceases locomotion. The contractile vacuole and nucleus assume a central position, or acquire such a little later on. The protoplasm is drawn together, and the cell gradually assumes the form of an oblate sphere literally studded with pseudopodia. When this stage is reached the nucleus can no longer be seen in the living cell. The reason for this will appear in the next section. When viewed by *reflected* light under a magnification of 30 diameters the hyaline area mentioned above is still visible lying apparently in the center of the cell. If the cell is now stimulated mechanically, as by transfer to a depression slide, etc., it will react. Pseudopodia will be extended, the spherical shape will be lost, and more or less normal locomotion will be resumed. After a period of a few minutes, however, the division sphere will again be formed and division will proceed normally. As time passes, the pseudopodia of the now fully formed division sphere become more uniform and finer until the cell takes on an appearance that may be compared to a chestnut burr or a rolled-up porcupine (Fig. 2). Reaction to shock is no longer possible after this stage is reached. After some minutes it will be noted that the pseudopodia, which throughout are being



Figs. 1–5 (legends on opposite page)

projected, withdrawn, and replaced by others, are gradually becoming coarser. It will also be seen that the hyaline area, hitherto clearly defined, is fading. Finally it disappears (Fig. 3), and shortly afterward the cell, now studded with relatively few large pseudopodia, rather suddenly loses its spherical shape and elongates (Fig. 4), usually in a plane parallel to the substrate. A sharp change soon appears, an irregular cleft forms at right angles to the axis of elongation, the pseudopodia attach to the substrate and actively draw the two daughter-cells apart, the bridge of protoplasm narrows to a thread (Fig. 5), snaps, and fission is completed.

It will be noted in the foregoing description that there is a gradual change in appearance throughout the process of division and that this change may be roughly divided as follows:

1. The initial stage in which the organism is not quite spherical, characterized by somewhat irregular pseudopodia and presence of the hyaline area.

2. The early stage of the division sphere characterized by fine pseudopodia and the presence of the hyaline area.

3. The late stage of the division sphere characterized by coarse pseudopodia and the disappearance of the hyaline area.

4. Elongation.

5. The formation of the cleft and completion of division.

These five stages are shown in Figures 1–5. These figures show, as well as the limitations of the medium and the skill of the artist will permit, the characteristic appearance of the organism under *reflected light*.

THE NUCLEAR PHENOMENA OF DIVISION

Observation of the living cell shows that in amoeba simultaneously with the formation of the division sphere the nucleus moves ap-

FIGS. 1–5.—Sketches showing the appearance of *Amoeba proteus* (Leidy) during division: (1) an amoeba just prior to the formation of the division sphere; (2) the same after completion of the sphere showing typical configuration during the early stages of division; (3) the same amoeba just prior to elongation of the cell—note the change in configuration; (4) the same amoeba elongating, the approximate position of the cleft is discernible; (5) the same amoeba when division is almost completed, the pseudopodia have nearly returned to the form typical of the cell between mitoses. NOTE.—The outlines of the sketches were made by means of a camera lucida and the detail filled in free hand from observation with reflected light, using a magnification of 30 diameters.

proximately to the center of the cell. The chromatin, which in the resting nucleus is distributed in granules or "blocks" immediately beneath the nuclear membrane (Fig. 6), leaves this position and moves toward the center of the nucleus. The karyosome becomes invisible, but the nuclear membrane can still be discerned until, as the rounding up is completed, the numerous granules and food vacuoles cover and prevent further observation of the nucleus. For information as to the subsequent changes, fixed and stained preparations must be relied on.

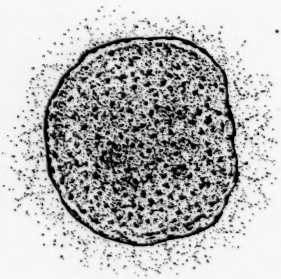


FIG. 6.—Camera lucida drawing of the normal resting nucleus in *Amoeba proteus* from mature individual; note that the chromatin is arranged just beneath the thick nuclear membrane, ×265.

Several series of such preparations have been made by us, and a typical series is presented in Figures 7–15, inclusive. Figure 7 shows the nucleus from a cell that has just become spherical. It will be seen that the chromatin has left the membrane and is evidencing a tendency to aggregate in a zone. In Figure 8, made a few minutes later, the chromatin appears completely withdrawn from the membrane and distributed through a network of more lightly staining apparently fibrilar material that may represent the karyosome. There is some tendency toward aggregation of the chromatin at the edge of this network which is roughly discoid, thus tending to form an irregular ring. Fibrillae extend from the chromatin at the periphery of this ring outward to the still intact nuclear membrane. The nucleus is apparently swollen. Surrounding the nucleus the cytoplasm appears less granular and forms *a vague hyaline area most pronounced next to the nucleus.*

In Figure 9 there is shown a still later stage. Here the chromatin has aggregated into a definite equatorial plate. In this figure it is seen almost from the edge. Examination of a number of slides has convinced us that this plate is in the form of a thin disç. The ground substance is granular and there are a few chromatin granules scattered through it, but the chromatin is mainly aggregated at the periphery.

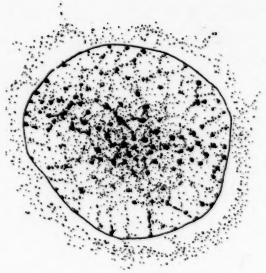


FIG. 7

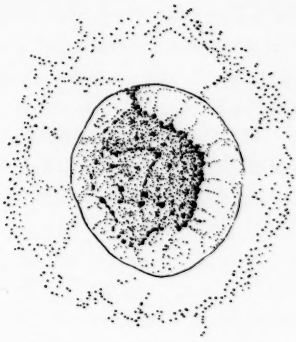


FIG. 8

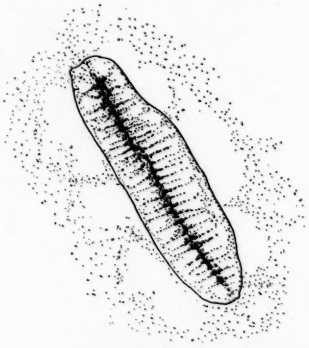


FIG. 9

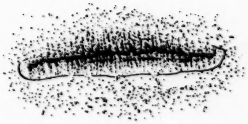


FIG 10

FIG. 7.—Camera lucida drawing of the nucleus in early prophase in *Amoeba proteus* showing the migration of the chromatin from the membrane and its incipient aggregation into a zone. Note vacuolization of cytoplasm about the nucleus. ×265.

FIG. 8.—Camera lucida drawing of the nucleus in mid-prophase in *Amoeba proteus* showing the beginning of plate formation and the condensation of the chromatin on the periphery of the plate together with the fibrillae extending from the chromatin to the nuclear membrane. Note the vacuolization of the cytoplasm adjacent to the nucleus. ×265.

FIG. 9.—Camera lucida drawing of the nucleus in late prophase in *Amoeba proteus* showing the completed equatorial plate apparently attached to the nuclear membrane by fibrillae. Note the condensation of chromatin at the periphery of the plate and the vacuolization of the cytoplasm about the nucleus. ×265.

FIG. 10.—Camera lucida drawing of the metaphase nucleus in *Amoeba proteus* seen in the plane of the equatorial plate. Note the disappearance of the nuclear membrane on the right face of the plate and lack of vacuolization of the cytoplasm on that face. ×265.

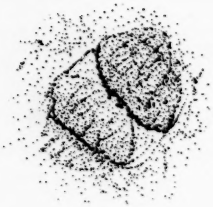


FIG. 11

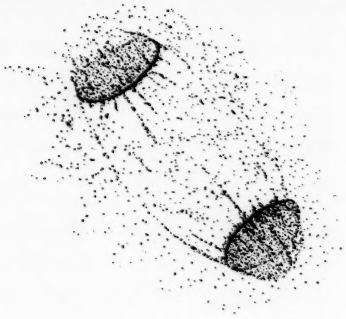


FIG. 12

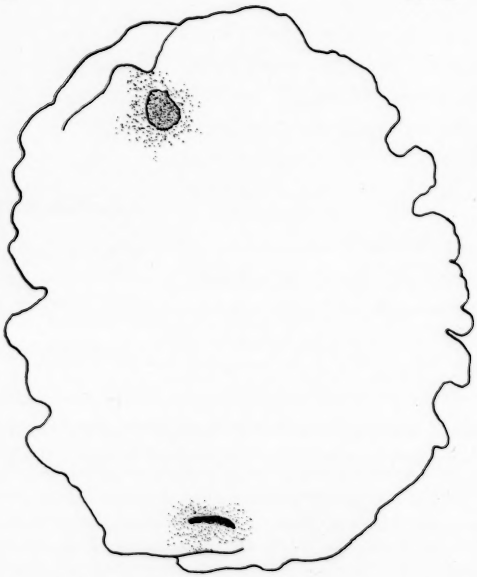


FIG. 13

FIG. 11.—Camera lucida drawing of the early anaphase nucleus in *Amoeba proteus* showing the daughter-plates just separated. Note faint spindle and polar caps and absence of vacuolization in cytoplasm. ×265.

FIG. 12.—Camera lucida drawing of the nucleus in mid-anaphase in *Amoeba proteus*. Note the distinct polar caps and faint spindle fibers between daughter-plates, that the chromatin on the periphery of the plates appears practically homogeneous, and the absence of vacuolization in cytoplasm about nucleus. ×265.

FIG. 13.—Camera lucida drawing of nuclei at elongation in *Amoeba proteus* showing their relation to the cell membrane. Note disappearance of spindle and caps, dense appearance of chromatin, and the close approximation of the nuclei to the cell membrane. ×130.

There are distinct clearly outlined fibrillae running outward from the plate to the still intact cell membrane. The entire nucleus, it will be seen, is markedly flattened in the plane of the ring. This stage marks the completion of the prophase.

Figure 10 shows the stage analogous to the metaphase of the metazoan cell. It is similar to the final prophase stage, except that the nuclear membrane is extremely faint and apparently entirely missing over one face of the plate.

FIG. 14

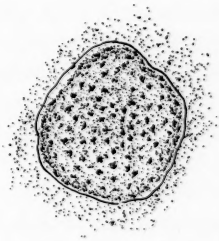


FIG. 15

FIG. 14.—Camera lucida drawing of the daughter-nuclei in mid-telophase in *Amoeba proteus* and their relation to the cell membrane showing the beginning of the reconstruction of the nuclei. Note the appearance of the new nuclear membranes and the maintained approximation of the nuclei to the cell membrane. ×130.

FIG. 15.—Camera lucida drawing of a daughter-nucleus in *Amoeba proteus* just after cell division is completed. Note complete reconstruction of nucleus. ×265.

Figure 11 shows the early anaphase. The nuclear membrane has now completely disappeared. The equatorial plate has become split and contracted in the plane of the plate resulting in the formation of two daughter-plates which have just separated. In some of our preparations there is visible a faint spindle formation. The more or less hyaline appearance of the surrounding cytoplasm has vanished.

In Figure 12 the plates are widely separated and are slightly less in diameter than in Figure 11. There are distinct polar caps of fibrillar material at the poles of the spindle.

Figure 13 shows the stage of elongation. The plates are in close proximity to the cell membrane, and the cell is elongating in the direction of the axis connecting the plates. This stage marks the end of the anaphase and the beginning of telophase.

Figure 14 shows a still later stage; the cleft that will separate the daughter-cells is well formed. The plates may still be seen close to the cell membrane, and about both a definite nuclear membrane is forming apparently thrown out from the plate.

Figure 15 shows the nucleus of a daughter-cell just separated. It is completely reconstituted.

It is recognized that this description is rough, but time is lacking at present for a complete cytological study of the process. It is hoped that one of us will be able later to carry out a more thorough investigation. In this regard it may be mentioned that the chromatin in the equatorial plate appeared as fine granules. If these represent the chromosomes they must be extremely numerous.

THE RELATION BETWEEN THE CYTOPLASMIC AND NUCLEAR PHENOMENA OF DIVISION

It is obvious from the above descriptions that there is a parallel series of phenomena exhibited by the cytoplasm on one hand and the nucleus on the other. It is also evident that there is probably a close correlation between the two series. It immediately occurred to us that if we could establish the degree of correlation it might be possible by selecting certain obvious cytoplasmic changes visible under low powers of magnification to select from culture, rapidly and accurately, any desired phase in the nuclear series, and thus provide the basis for a technique which would permit physiological study of the separate nuclear phases of the division process.

We accordingly attempted to ascertian the time-relation of the various cytoplasmic changes and the time-relation of the nuclear changes in order that their temporal correspondence might become evident. We first studied the time-relations of the cytoplasmic (form, etc.) changes. The transformations of form are gradual. It

was therefore necessary to select definite points from which or to which measurement of time-intervals could be made with a reasonable degree of accuracy. These points must obviously be the observable phenomena in the series which have a comparatively high rate of change thus permitting their position in the series to be fairly accurately ascertained. We selected the following: (1) the attainment of the spherical form; (2) the disappearance of the hyaline area; (3) the loss of spherical form (elongation); (4) the actual separation of the daughter-cells.

The attainment of sphericity by the cell was at first (due to our inexperience) rarely observed; hence in our first measurements we used mainly the disappearance of the hyaline area, elongation, and the moment of separation of the daughter-cells. Measurements of the time-intervals elapsing between these changes and the time when the amoeba was first observed, or when it attained sphericity, were made on 45 animals. From the measurements thus made we found for each amoeba the interval from the first observation to the disappearance of the hyaline area (if present), the interval from attainment of sphericity to the disappearance of the area when such attainment was observed, the interval from the disappearance of the area to elongation, the interval from elongation to the separation of the daughter-cells, and the interval from the first observation, or attainment of sphericity, to elongation. All measurements were made at an average temperature of 24° C., the range of temperature being from 22° to 25° C. The amoebae were observed *in situ* in culture under a binocular dissecting microscope until shortly after the disappearance of the hyaline area or in some instances just after elongation. Then they were carefully transferred to a depression slide and the observation of the moment of separation ascertained, using a compound microscope with 20X ocular and 8 mm. objective. A water cell was placed between the light source and the microscope to obviate temperature changes. The data thus obtained are presented in Table I. From this table it will be seen that the average time from the attainment of sphericity to elongation in the cases in which this was observed was 24 minutes, with a *range* from 19 to 28 minutes. The average interval from the disappearance of the hyaline area to elongation was 6.9 minutes with a *range* from 4 to 12 minutes. The

TABLE I

TABLE SHOWING THE INTERVALS (IN MINUTES AND AT 24° C.) BETWEEN THE VISIBLE CYTOPLASMIC CHANGES IN 45 DIVIDING AMOEBAE

No.	Condition of Hyaline Area + Present ± Doubtful − Absent	Time from First Observation to Disappearance of Hyaline Area	Time from Completion of Division Sphere to Disappearance of Hyaline Area	Time from Disappearance of Hyaline Area to Elongation	Time from Elongation to Division	Time from Completion of Division Sphere to Elongation	Time from First Observation to Elongation, Hyaline Area Present	Time from First Observation to Elongation, Hyaline Area Doubtful	Time from First Observation to Elongation, Hyaline Area Absent
1	±	.....	.....	.....	7	.....	.....	10	.....
2	±	.....	.....	.....	5	.....	.....	12	.....
3	+	15	.....	9	6	.....	24	.....	.....
4	+*	17	17	9	16	26	.....	.....	.....
5	−	.....	.....	.....	12	.....	.....	.....	1
6	+	16	.....	8	6	.....	24	.....	.....
7	+*	18	18	7	5	27	.....	.....	.....
8	+	17	.....	4	9	.....	21	.....	.....
9	±	3	.....	6	6	.....	.....	9	.....
10	−	.....	.....	.....	9	.....	.....	.....	7
11	+	7	.....	4	7	.....	11	.....	.....
12	±	6	.....	7	9	.....	.....	13	.....
13	+	9	.....	8	9	.....	17	.....	.....
14	−	.....	.....	.....	9	.....	.....	.....	9
15	+	13	.....	5	8	.....	18	.....	.....
16	−	.....	.....	.....	9	.....	.....	.....	1
17	+	13	.....	4	.....	.....	17	.....	.....
18	±	.....	.....	6	(27†)	.....	.....	14	.....
19	+*	18	18	10	8	28	.....	.....	.....
20	+	5	.....	8	9	.....	13	.....	.....
21	+	18	.....	7	10	.....	18	.....	.....
22	−	.....	.....	.....	10	.....	.....	.....	9
23	+*	7	7	5	.....	.....	12	.....	.....
24	+	19	.....	5	14	.....	24	.....	.....
25	−	.....	.....	.....	7	.....	.....	.....	6
26	+	6	.....	7	8	.....	15	.....	.....
27	+*	18	18	4	10	22	.....	.....	.....
28	+	6	.....	6	9	.....	13	.....	.....
29	+	11	.....	6	9	.....	17	.....	.....
30	+*	11	11	8	7	19	.....	.....	.....
31	+	5	.....	9	13	.....	14	.....	.....
32	+	13	.....	4	13	.....	17	.....	.....
33	+	13	.....	8	8	.....	21	.....	.....
34	−	.....	.....	.....	15	.....	.....	.....	2
35	+	7	.....	7	7	.....	14	.....	.....
36	+	13	.....	8	7	.....	21	.....	.....
37	±	.....	.....	.....	6	.....	.....	7	.....
38	+	10	.....	12	10	.....	22	.....	.....
39	+*	12	12	10	10	22	.....	.....	.....
40	−	.....	.....	.....	6	.....	.....	.....	6
41	+	14	.....	8	8	.....	19	.....	.....
42	+	10	.....	7	7	.....	17	.....	.....
43	−	.....	.....	.....	5	.....	.....	.....	7
44	±	.....	.....	.....	7	.....	.....	4	.....
45	+	2	.....	5	5	.....	7	.....	.....
Average	.....	11.3	15.0	6.9	8.5	24	17.6	9.8	5.3

* Individuals seen to form division sphere.

† This amoeba gave a very poor and unequal telophase and was not included in calculation of the average for the interval from elongation to division.

average interval from elongation to separation of the daughter-cells was 8.5 minutes with a *range* from 5 to 14 minutes. It will further be noted that for the amoebae, in which the hyaline area was absent when first observed, the average interval from the first observation to elongation was 5.5 minutes, i.e., less than the average interval between the disappearance of the area and elongation. When the hyaline area was doubtful, the average interval was 9.8 minutes, i.e., slightly greater than the interval from the disappearance of the area

TABLE II

TABLE SHOWING ELAPSED TIME FROM COMPLETION OF DIVISION SPHERE TO ELONGATION IN 10 AMOEBAE

No.	Time Division Sphere Completed	Calculated Time of Elongation	Observed Time of Elongation	Elapsed Time to Elongation (Minutes)	Difference in Observed and Calculated Time	Temperature of Culture (° C.)
1	2:37	3:02	3:02	25	00	23
2	3:48	4:13	4:07	19	06	25
3	9:28	9:53	9:54	26	01	24
4	10:03	10:28	10:28	25	00	25
5	10:56	11:21	11:23	27	02	25
6	11:49	12:14	12:13	24	01	25
7	11:58	12:23	12:21	22	02	25
8	9:54	10:19	10:23	28	05	22
9	1:27	1:52	1:58	29	06	22
10	2:05	2:30	2:28	23	02	23
Average	.....	.....	.....	24.8	2.5	23.9

to elongation. When the hyaline area was present but the attainment of sphericity was not observed, the average interval from the first observation to elongation was 17.6 minutes, i.e., a longer interval than when the area was doubtful or absent, but shorter than when the cell was seen to attain the spherical form. We felt that these data were sufficient to establish definitely the interval from the disappearance of the area to elongation and the interval from elongation to division, but we desired further data on the relation of the attainment of sphericity to these. Therefore, we measured the interval from attainment of sphericity to elongation in 10 other amoebae, noting the temperature in each instance; the result is presented in Table II. It will be seen that the average interval is 24.8

and the range from 19 to 29 minutes. Thus we can set the average interval definitely as between 24–25 minutes at the temperature of approximately 24° C. The most striking fact in the entire series of measurements shown in Tables I and II is their remarkable uniformity.

While making these measurements we noticed that the coarsening of the pseudopodia (compare Figs. 2 and 3) apparently begins shortly before the disappearance of the hyaline area. We therefore decided to attempt to measure this change in order to obtain a further quantitative expression of the cytoplasmic change during cell division. To do this we made measurements of the width of the individual pseudopodia on 17 amoebae, using a compound microscope with 20X ocular and 4 mm. objective, together with an eyepiece micrometer. The measurements were made as follows: Each amoeba was taken from the culture together with a small quantity of fluid by means of a capillary pipette, placed in a shallow cell, made from strips of cover glass cemented to a microscope slide and covered with a cover glass. Then, with the microscope so focused as to give a median optical section of the cell, the width of each pseudopodium was measured. Endeavor was made to ascertain the width of all pseudopodia seen in the section within the interval of 1 minute. The time at which measurements were begun and ended, together with the measurements, was recorded, thus giving a series of measurements and its limits in time. This procedure was repeated, sometimes with no interval allowed between the series of measurements, but usually an interval of a minute or so was allowed. The measurements were thus made at intervals on each animal until elongation occurred. No measurements beyond elongation would be significant as after this the pseudopodia assume the characteristics exhibited during the intermitotic phase, i.e., they are quite variable in width and show great variation in form.

The individual series of measurements for each amoeba were separately averaged and the averages plotted against the midinterval of each series. We thus obtained a curve for each amoeba showing the changes in the average width of the pseudopodia during the period of observation. Three such curves are presented in Figure 16. Two of these, delineated in solid lines, were selected as

typical. One delineated with a dotted line is the extreme deviation found. It is presented in order to give an idea of the amount of varia-

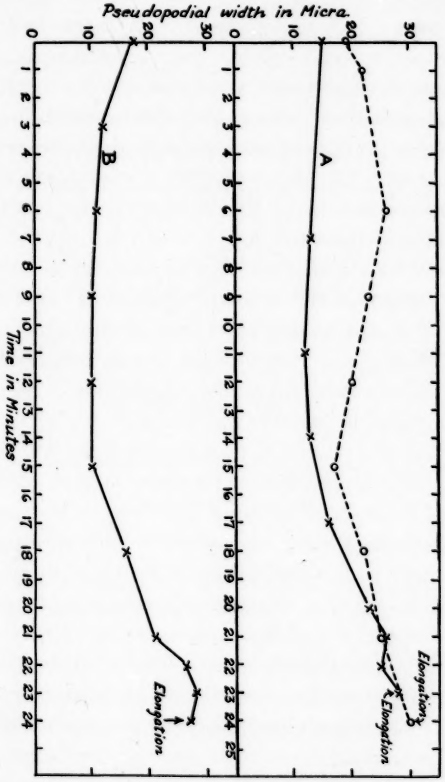


FIG. 16.—Graph showing the changes in the average width of the pseudopodia of three division spheres of *Amoeba proteus*, the two curves drawn with solid lines are typical. The dotted line gives the most atypical curve obtained. Each point on the curve is the average of the measured widths of 15–20 pseudopodia. Note that an increase in average width begins in each curve 9 minutes before elongation; note also the smoothness of the individual curves.

tion in the course of these curves. It will be noted that the two typical curves show initially wide pseudopodia; their width rapidly decreases and becomes constant until about 9 minutes prior to

elongation, when an increase in width begins which continues to elongation. The atypical curve differs from the others particularly in the increase in average pseudopodial width, which reached a maximum about 6 minutes after the sphere was formed, followed by a gradual decrease until about 9 minutes before elongation. This fluctuation in average width was the result of the extrusion and subsequent withdrawal of two or three unusually wide pseudopodia. If these had been eliminated the course of the curve would be typical.

To include here the great number of individual measurements of the pseudopodia would be to overburden the reader, but in order that some idea of the variation in width in the pseudopodia may be given, it may be stated that in a typical set of measurements the average deviations of the measurements at 24, 21, 12, and 9 minutes prior to, and at, elongation were respectively 35, 25, 30, 10, and 22 per cent of their mean values. This would indicate that in addition to the fluctuation in the average width of the pseudopodia, there occurs *pari passu* a variation in the amount of uniformity of the width which attains its maximum during the period when the width is minimum. Finally, all curves for the change in pseudopodial width were plotted so as to make their moments of elongation coincident and the entire data were averaged for each minute interval. The composite curve thus produced is presented in Figure 17. From this figure it will be seen that the curve covers the 25 minutes immediately prior to elongation and thus covers the average interval from the initial formation of the division sphere ("attainment of spherical shape") to elongation (compare Table II). Further, it will be noted that at the beginning of this period the average width of the pseudopodia is approximately 18 micra, during the next 6 minutes it decreases to 13 micra. It then remains relatively constant for the 10 succeeding minutes when it begins to increase and continues to do so for about 9 minutes, at the expiration of which interval elongation occurs. It is significant to note that the interval between the beginning of this increase in width and elongation is practically the same as that found betwen the disappearance of the hyaline area and elongation (see Table I).

We were not able to ascertain with certainty in the living cell the course of the functioning of the contractile vacuole because its out-

line was so obscured and broken up by the thick layer of granules which surrounded it in the division sphere. However, in 85 stained preparations, 42 of which showed the nucleus in prophase, 23 in anaphase, and 20 in telophase, contractile vacuoles were present in 22 of the 42 prophase preparations, but were found in none of those showing anaphase or telophase nuclei. We conclude from this that functioning of the contractile vacuole ceases at about metaphase and is not resumed until at least very late telophase.

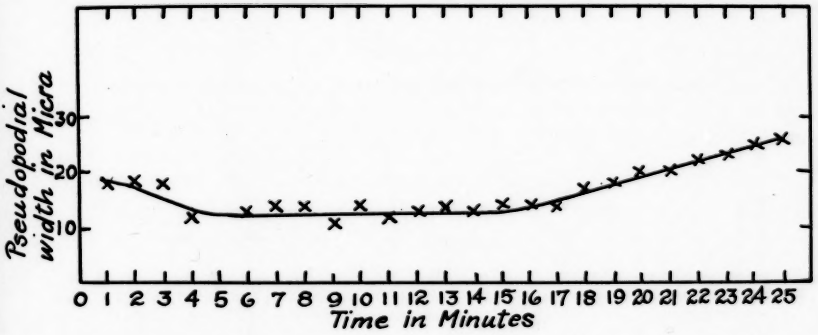


FIG. 17.—Graph showing the change in average width of the pseudopodia of the "division sphere" of *Amoeba proteus* based on measurements made on 17 amoebae from the formation of the sphere to elongation. Note the short initial decrease and the final increase in average width beginning about 9 minutes before elongation.

We now turned to the time-relation of the nuclear changes. To ascertain this we selected 30 amoebae that were observed to form their division spheres. One lot of 5 of these was fixed and stained as soon as each amoeba formed its sphere, and similar lots were fixed and stained 5, 10, 15, 20, and 25 minutes after formation of the spheres.

The nuclear conditions found in these lots are shown in Table III. From this it is at once apparent that during the first 10 minutes following formation of the sphere the nucleus is in prophase; during the next 5 the metaphase stage is reached; and during the 10 minutes until elongation occurs the anaphase stages are completed.

Having obtained the time-relations of both the nuclear and cytoplasmic changes we could now by plotting all data on one graph

show their degree of correlation. The combined results are presented in Figure 18. From this figure it will be seen that the typical course of events is as follows: When the division sphere is formed, the hyaline area is clearly visible, the contractile vacuole is active. The pseudopodia have an average width of about 18 micra and the nucleus has just entered prophase. The outward appearance at this time is fairly well presented in Figure 1, which in fact shows a slightly earlier stage. After 5 minutes the hyaline area and contractile vacuole are unchanged, but the pseudopodia now average only about

TABLE III

TABLE SHOWING TIME-DISTRIBUTION OF VARIOUS NUCLEAR PHASES IN *Amoeba proteus* (LEIDY) AT A TEMPERATURE OF 24° C.

	Prophase			Anaphase			Telophase
	Early	Mid	Late	Early	Mid	Late	
Just completed division sphere	6						
5 minutes	1	5					
10 minutes		1	3				
15 minutes				1	3	1	
20 minutes					1	4	
25 minutes					1	2	2

13 micra in width and the nucleus has progressed so far in the prophase that the chromatin blocks have begun to form a plate. At the end of 10 minutes there is no change in the hyaline area, the contractile vacuole, or the pseudopodial width, but the nucleus is practically at the end of prophase with the chromatin fully formed into the equatorial plate but still inclosed in the nuclear membrane. The external appearance is that shown in Figure 2.

Sometime during the next 5 minutes the nuclear membrane vanishes; this nuclear phase we regard as analogous to the metaphase in metazoan cells. At the end of that time, 15 minutes after the formation of the sphere, the hyaline area has become much fainter and no discernible change has taken place in the pseudopodia, but there is no longer a contractile vacuole, and the two daughter-plates of chromatin have begun to separate.

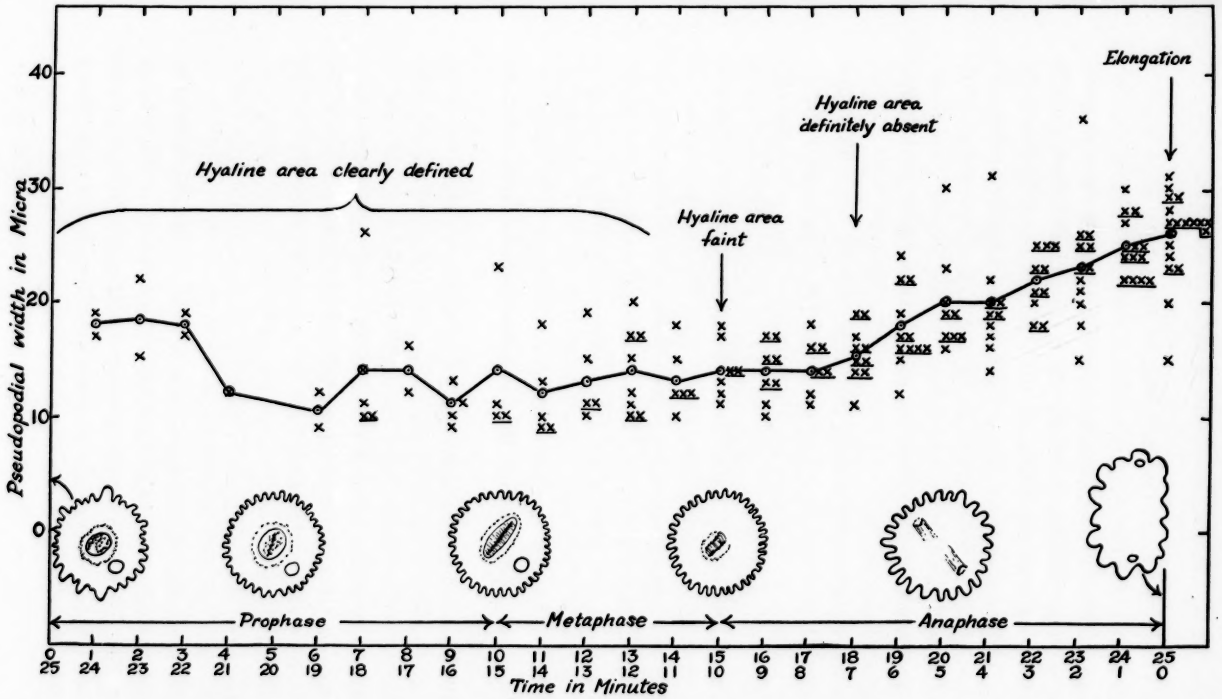

Elongation
Hyaline area definitely absent
Hyaline area clearly defined
Hyaline area faint
Pseudopodial width in Micra
40
30
20
10
0
Prophase
Metaphase
Anaphase
Time in Minutes
0 25 1 24 2 23 3 22 4 21 5 20 6 19 7 18 8 17 9 16 10 15 11 14 12 13 13 12 14 11 15 10 16 9 17 8 18 7 19 6 20 5 21 4 22 3 23 2 24 1 25 0

FIG. 18.—Graph showing the correlation between the cytoplasmic and nuclear changes during division in *Amoeba proteus*. The solid curve shows the change in the average width of the pseudopodia from the formation of the division sphere to elongation. The points indicated by X give the average widths for the individual amoebae, all points in underlined groups have the co-ordinates of the left-hand point of the group. The diagrams in the lower portion of the graph indicate the typical condition of the hyaline area, nucleus, pseudopodia, and contractile vacuole at the end of each 5-minute period from the formation of the sphere to elongation.

After 20 minutes from the formation of the sphere have elapsed, the hyaline area has completely vanished, as has of course the contractile vacuole. The pseudopodia have increased in average width to 20 micra and the two daughter chromatin plates are separated by a distance at least two-thirds the diameter of the cell. Lastly, at the expiration of 25 minutes the cell begins to elongate; at this time the daughter-plates lie at the ends of the axis of elongation close beneath the cell membrane, and the pseudopodia average approximately 27 micra in width. Figure 4 gives a fair idea of the external appearance of this stage.

As we have previously stated, from the time of elongation until actual separation of the daughter-cells a period of approximately 8.5 minutes elapses. It has also been pointed out that measurements of the width of the pseudopodia from here on are of little importance. We did not, therefore, include this last stage in Figure 18. The phenomena that occur during this last period may be summarized as follows: A cleft appears at right angles to the axis of elongation and deepens until the daughter-cells finally separate. During the period just prior to actual separation protoplasmic streaming occurs—a current flowing at short intervals alternately from one daughter-cell to the other. The new nuclear membrane develops. The daughter-nuclei swell somewhat and at the time separation occurs are completely formed.

It will be seen that as a consequence of this correlation we are in position to fix criteria by which the nuclear condition of the cell may be determined from its external aspect. The prophase of the nucleus is indicated externally by fine pseudopodia and the presence of a *distinct* hyaline area, the metaphase by fine pseudopodia and a *very faint* hyaline area, and the anaphase by coarse pseudopodia and the *absence* of the hyaline area. Telophase, of course, presents no difficulties since the cell during this entire period is marked by its loss of sphericity and the cleft formation.

We now proceeded to test the accuracy of these criteria. It will be noted that the *only* index of metaphase is the *faintness* of the hyaline area and we had reason to think that the actual duration of this phase is very short. Hence we did not expect any success in actually selecting individuals at metaphase. Telophase, on the other hand,

could evidently be selected with absolute certainty. The real problem was to ascertain how accurately our criteria would enable us to select prophase and anaphase nuclei. We selected on the basis of the criteria mentioned 17 division spheres as containing prophase, and 13 as containing anaphase, nuclei. None of these spheres was observed to form, and the selection was made from culture under a magnification of 30 diameters. Each amoeba selected was immediately fixed and the cover glass labeled prophase or anaphase as the criteria indicated. All prophase and all anaphase selections were further kept separate until stained and examined. The results are presented in Table IV. As between anaphase and prophase it will be

TABLE IV

TABLE SHOWING THE DISTRIBUTION OF NUCLEAR PHASES IN TWO SELECTED GROUPS OF DIVIDING AMOEBAE

	Prophase			Anaphase			Telophase
	Early	Mid	Late	Early	Mid	Late	
Selected for prophase nuclei...	8	4	4	1	0	0	0
Selected for anaphase nuclei...	.....	.....	.....	1	7	5	0

seen the selection shows an error of 3.33 per cent. We then attempted to select for the metaphase stage in a similar manner, using 10 amoebae, not with the expectation of accurately chosing the metaphase stage, but of getting a distribution that was fairly evenly divided between prophase and anaphase. The results of this trial showed 6 early anaphase, and 4 prophase, nuclei.

It may be well to point out that, since the selection of the different phases is based on the evaluation of morphologic criteria, we can confidently expect an increase in the accuracy of selection with greater experience.

In concluding this section it may be of interest to note that the volumes of 25 of the division spheres were measured and the results presented in Figure 19. This figure shows that the average volume was 0.00264 cubic millimeter, the range was from 0.0022 to 0.0032

with the mode at 0.00270. This is in good agreement with the value arrived at by statistical methods by Chalkley (1931) of 0.00275 cubic millimeter, which he suggested as an optimum volume for division. Only 2 of the 100 or more division spheres watched during

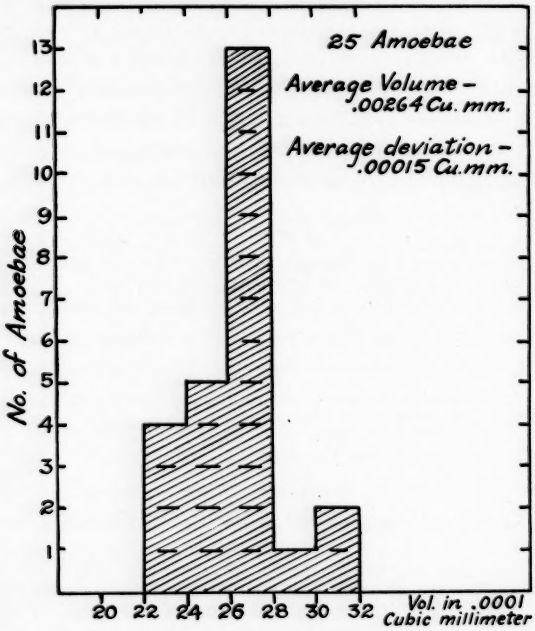


FIG. 19.—Graph showing the distribution in volume of 25 "division spheres" of *Amoeba proteus*. Note that this distribution indicates strongly an "optimum volume" of 0.0027 cubic millimeter.

the course of the investigation failed to divide, except when the process was deliberately stopped. In both of these a binucleate form was produced due to failure of cytoplasmic division. In one instance a division sphere of rather large size gave rise to three individuals; examination of their nuclei showed one of these to be binucleate and two mononucleate, indicating their derivation from an originally binucleate amoeba.

DISCUSSION

In considering the data presented, two aspects seem to merit discussion—the implications of the data from the physiological standpoint, and their relation to the prior investigations on division in this organism.

We shall first deal with the physiological aspect. Obviously the great uniformity of the temporal series of phenomena both in nucleus and cytoplasm indicates clearly that we are dealing with organisms exhibiting a remarkable uniformity of physiologic state. This appears to us to be of considerable value since it indicates that by using individuals in this state, if adapted to the particular experimental purposes, we can minimize to a great extent the error normally encountered as a result of individual variation.

Certain temporal correlations in the series of changes that are presented also seem to possess considerable physiological significance. We refer to the time-relation that exists between the beginning of the change in the pseudopodia prior to elongation, the cessation of the action of the contractile vacuole, and the disappearance of the nuclear membrane. This relation is clearly presented in Figure 18. This shows that the disappearance of the nuclear membrane precedes shortly the beginning of the change in the pseudopodia and apparently is approximately coincident with the cessation of activity of the contractile vacuole. The widening of the pseudopodia, it will be noted from Figure 3, is accompanied by a deepening of the clefts between them, and this would probably mean an increase in cell surface which would seem to connote a lowering of surface tension. The cessation of the function of the vacuole which is generally held to be a regulator of the water content of the organism would also seem to indicate an increase in water content of the cell. It is widely held that a change in surface tension with respect to the surrounding medium plays a considerable rôle in bringing about the division of the cytoplasm. It has been suggested by Ruzicka (1922) and others that senility in the organism arises as a consequence of a decrease of dispersal, "Verdichtung," of the colloidal constituents. Further, in regard to the particular organism under consideration, Chalkley (1931) noted that prior to division the

cells appear densely granular and sluggish, while immediately after they are hyaline and active.

These considerations, together with the facts presented, seem to us to indicate the possibility that at metaphase there is released from the nucleus into the cytoplasm a substance or substances which so act as to lower surface tension and produce greater hydration of the cytoplasmic colloids, thus assisting in cytoplasmic division and leading toward rejuvenation of the cell in so far as the latter is conditioned by the hydration of its colloidal constituents. We believe that by making use of a technique based on the facts here presented it should not be extremely difficult to discover whether release of the nuclear content into the cytoplasm does in fact result in increased cell surface at least, and whether a definitely higher water content exists in the cell after the metaphase is passed. We hope that experimentation will be made in this direction.

Finally, we wish to emphasize that the ability to select readily under low powers of magnification cells in any desired stage of nuclear division, metaphase excepted, should greatly simplify the experimental study of nuclear division.

We now come to the question of the diverse interpretations of the fission process mentioned in the introduction. The observations of Awerinzew (1904) and Carter (1912), while in accord in their major outlines with the picture presented here, were certainly made not on *Amoeba proteus* (Leidy) but probably on *Amoeba dubia* (Sch.), since both the authors state that the resting nucleus was spherical or ovoid. The resting nucleus in *proteus* is *discoid*. The report of Levy (1924) stating that he could find only scattered chromatin granules in the division spheres he examined requires explanation since he undoubtedly worked with *Amoeba proteus*. We at first attempted to stain with Heidenhain's haematoxylin. Heidenhain's stain, at least in our hands, stained not only the nuclear material but other very numerous granules scattered in the cytoplasm, which granules obscured the nucleus almost completely. On destaining, both the nuclear chromatin and granules destained, the latter the least rapidly, and when the process was carried to the point where it would seem reasonable to expect to see the nucleus, it could not be distinguished but the granules were still sharply stained. These

granules were doubtless those described by Levy. We confirm Doflein (1918) entirely as to the nuclear changes in division. We venture to differ on one point. We have, as pointed out, seen only one instance of multiple fission in several hundred watched divisions. Further, one of us (C.), during the last two years, has noted the division of something like one thousand isolated amoebae under survival conditions and has found multiple fission to be quite infrequent. In regard to the illustrations given by Doflein of bi- and polynucleate division spheres, all of these show the nuclei as *resting nuclei*. It appears to us that this indicates that what Doflein believed to be division spheres are not such at all, but merely rounded up resting polynucleate amoebae. These occur more or less frequently, especially in old cultures. We are further lead to infer this confusion from the fact that he states that the division sphere "entweder ovoid oder volkommen kugelig, glattrandig oder mit eigenartig regelmässigen Protoplasmafortsätzen ringsum besetzt ist." We have never at any time seen a smooth-surfaced rounded-up amoeba divide. The pseudopodia-studded spherical cell is the only one in our experience that has divided. On the contrary, when in a few instances non-typical spheroidal forms were accidentally fixed and stained, we have found invariably that they contained resting nuclei. We must therefore differ with Doflein in respect to the typical vegetative reproduction in *Amoeba proteus*. As far as we can ascertain it is binary fission. Occasionally there is failure of cytoplasmic division leading to the formation of polynucleates as we have observed. These may later divide. Such a process, however, does not appear to be completely normal.

In respect to the peculiar nuclear forms observed by Taylor (1923) we are at present in doubt as to whether they represent another type of division. We have made numerous observations on these forms but have as yet reached no definite conclusion as to their origin or fate.

SUMMARY

Amoeba proteus normally divides by binary fission. The nucleus divides promitotically and the nuclear membrane disappears at metaphase. The average time for the division process at a temperature of 24° C. is 33 minutes, with an average deviation of about 2

minutes. The prophase lasts about 10 minutes, the metaphase not over 5 minutes, probably less, the anaphase about 10 minutes, and the telophase about 8 minutes. The nuclear phases are accompanied by typical changes in cell form that are readily distinguishable under a magnification of 30 diameters. During prophase the cell is spherical, studded with fine pseudopodia, and exhibits under reflected light a clearly defined hyaline area at its center. During metaphase the picture is similar, except that the hyaline area is very faint. During anaphase the pseudopodia become rapidly coarser, and the hyaline area is no longer visible. The telophase is accompanied by elongation of the cell and cleft formation.

Under the cultural conditions used, the average measured width of the pseudopodia in early prophase was 18 micra; from midprophase to metaphase it was 13–14 micra. At anaphase the average width began to increase and at the beginning of telophase attained a value of 26 micra. It is possible merely from observation of the external appearance of the living cell to select from culture under a magnification as low as 30 diameters cells showing any desired stage of *nuclear* division except actual metaphase. Such selection can readily be made with 97 per cent accuracy, thus making possible the study of the effect of reagents upon the separate nuclear phases in cell division. The modal volume of *Amoeba proteus* at division under the cultural conditions employed is 0.0027 cubic millimeters. Binucleate forms are produced in culture by failure of cytoplasmic fission. Evidence is presented that the contractile vacuole ceases to function during division from the time that the nucleus enters the metaphase stage until cell division is completed. The close correlation of the disappearance of the nuclear membrane with the cessation of the activity of the contractile vacuole and the beginning of the rapid increase in average width of the pseudopodia during the nuclear anaphase suggests a causative relation between these phenomena.

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